RESEARCH ARTICLE

GmPIN-dependent polar auxin transport is involved in soybean nodule development

Zhen Gao[1,2,#], Zhiwei Chen[1,2,#], Yuanyuan Cui[1,2,#], Meiyu Ke[2,3,#], Huifang Xu[2,3], Qinzhen Xu[4,5], Jiaomei Chen[2], Yang Li[6], Laimei Huang[2], Hong Zhao[2], Dingquan Huang[2], Siyuan Mai[2,3], Tao Xu[2,3], Xiao Liu[4,5], Shujia Li[7], Yuefeng Guan[2], Wenqiang Yang[4,5], Jiří Friml[8], Jan Petrášek[9], Jing Zhang[6] and Xu Chen[2,*]

[1] College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.
[2] Haixia Institute of Science and Technology, Horticultural Plant Biology and Metabolomics Center, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.
[3] College of Life Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China.
[4] Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China.
[5] University of Chinese Academy of Sciences, Beijing 100049, China.
[6] State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China.
[7] State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China.
[8] Institute of Science and Technology Austria (IST Austria), Am Campus 1, 3400 Klosterneuburg, Austria.
[9] Institute of Experimental Botany of the Czech Academy of Sciences, Prague, Czech Republic.

* Correspondence: Xu Chen (chenxu@fafu.edu.cn)
# These authors contributed equally to this work.

Short title: Auxin transport regulates soybean nodule formation

One-sentence summary: In soybean, nodule primordium formation involves GmPIN1-mediated polar auxin transport within primordium cells, and nodule enlargement involves the collaboration of GmPIN9d and GmPIN1-dependent auxin transport within nodule vasculature.

1   **ABSTRACT**

2   To overcome nitrogen deficiency, legume roots establish symbiotic interactions with nitrogen-

3   fixing rhizobia that is fostered in specialized organs (nodules). Similar to other organs, nodule

4   formation is determined by a local maximum of the phytohormone auxin at the primordium

5   site. However, how auxin regulates nodule development remains poorly understood. Here, we

6   found that in soybean, (*Glycine max*), dynamic auxin transport driven by PIN-FORMED (PIN)

7   transporter GmPIN1 is involved in nodule primordium formation. *GmPIN1* was specifically

8   expressed in nodule primordium cells and GmPIN1 was polarly localized in these cells. Two

9   nodulation regulators, (iso)flavonoids trigger expanded distribution of GmPIN1b to root

10   cortical cells, and cytokinin rearranges GmPIN1b polarity. *Gmpin1abc* triple mutants

11   generated with CRISPR-Cas9 showed impaired establishment of auxin maxima in nodule

12   meristems and aberrant divisions in the nodule primordium cells. Moreover, overexpression of

13   *GmPIN1* suppressed nodule primordium initiation. GmPIN9d, an ortholog of *Arabidopsis*

14   *thaliana* PIN2, acts together with GmPIN1 later in nodule development to acropetally transport

15   auxin in vascular bundles, fine-tuning the auxin supply for nodule enlargement. Our findings

16   reveal how PIN-dependent auxin transport modulates different aspects of soybean nodule

17   development and suggest that establishment of auxin gradient is a prerequisite for the proper

18   interaction between legumes and rhizobia.

19

20   **INTRODUCTION**

21   The developmental plasticity of plant cells depends largely on de-novo post-embryonic

22   generation of new organs initiated from specialized tissues (Benkova et al., 2003; Yamaguchi

23   et al., 2013; Qi et al., 2014). For example, a lateral root is generated from pericycle cells, via a

24   series of anticlinal divisions, giving rise to lateral root primordia (Benkova and Bielach, 2010).

25   Leaf bulges formed at the flanks of the shoot apical meristem generate leaf primordia (Xiong

26   and Jiao, 2019). Some primordia differentiate into determinate organs with constant cell

27   numbers, whereas others develop into indeterminate organs with a secondary meristem at the

28   tip.

29   Initiation and positioning of an organ primordium is often instructed by local

30   accumulation of the phytohormone auxin (Benkova et al., 2003; Qi et al., 2014; Wu et al.,

31   2015). Local auxin accumulation within founder cells depends mainly on the coordinated

32   activities of AUXIN1/LIKE-AUXIN1 influx carriers and PIN-FORMED (PIN) efflux carriers

33   in conjunction with other auxin transporters (Mravec et al., 2008). The roles of different PINs

34   are well characterized in the context of auxin transport for organ growth and development in

35 *Arabidopsis thaliana*, such as PIN1 in flower development (Okada et al., 1991), PIN2 in root

36 gravitropism (Muller et al., 1998), PIN3 in shoot tropism (Friml et al., 2002), PIN4 and PIN7

37 in embryo development (Benkova et al., 2003; Weijers et al., 2005).

38   The cellular polarity of PIN proteins is tightly related to the direction of auxin flow.

39 For example, primary root growth requires the acropetal/rootward direction of auxin transport

40 by PIN1 which targets the basal side of the plasma membrane (PM) in stele cells, and the

41 basipetal/shootward direction of auxin transport by PIN2 that targets the apical side of PM in

42 epidermal cells, as well as the coordination of PIN3, PIN4 and PIN7 for auxin lateralization

43 within root stem cells (Feraru and Friml, 2008). Current models in diverse organ patterning

44 explicitly show that a PIN-dependent local auxin gradient participates in nearly all types of

45 organ development processes in plants (Benkova et al., 2003; Wisniewska et al., 2006).

46   Many legumes (Fabaceae) are capable of symbiotic interaction with soil bacteria (rhizobia)

47 in specialized organs, called nitrogen-fixing root nodules. The initiation of nodules is triggered

48 by specific signaling molecules, lipochitooligosaccharides (LCOs)/Nod factor released by

49 rhizobia, in response to flavonoids secreted from the root hairs of host plants (Suzaki and

50 Kawaguchi, 2014). Subsequently, the rhizobia penetrate the root via an infection thread,

51 reaching the cortex cells through the curled root hairs. In the cortical cells, the rhizobia

52 stimulate cell proliferation and expansion, resulting in a functional nodule on the host root

53 (Popp and Ott, 2011). Later, the entire nodule is surrounding by a continuum of vascular

54 bundles, providing a robust route to exchange water and organic materials between the root

55 and nodule (Livingston et al., 2019). Within the nodule, rhizobia reside as intracellular

56 symbionts, where they convert atmospheric nitrogen into ammonia to overcome nitrogen

57 shortage in the host plant.

58   Different legume species show substantial variation in nodule organogenesis and nodules

59 are broadly defined as indeterminate versus determinate types. *Medicago truncatula* develops

60 indeterminate nodules, which harbor active apical meristems that continuously produce new

61 meristem tissue. *Lotus japonicus* and *Glycine max* (soybean) generate determinate nodules,

62 whose meristematic activity is lost soon after their initiation (Popp and Ott, 2011). Irrespective

63 of nodule type, these nodules all initiate from a primordium and subsequently develop into

64 diverse organ morphologies. Auxin accumulates at the position of future primordia, to

65 prepare/prime the initiation of nodule primordia (van Noorden et al., 2007; Suzaki et al., 2012;

66 Turner et al., 2013). In soybean, accumulating evidence further illustrates the importance of

67 auxin for nodule development. For example, overexpression of a nodule-expressed auxin

68 biosynthesis enzyme *YUCCA2a (GmYUC2a)* delays nodule organogenesis (Wang et al., 2019).

69 Moreover, overexpression of the auxin receptor *TRANSPORT INHIBITOR RESPONSE1*
70 *(GmTIR1)* and suppression of *AUXIN RESPONSE FACTOR8 (GmARF8)* increases rhizobia
71 infection events and nodule number (Wang et al., 2015b; Cai et al., 2017). Besides many
72 similarities between determinate nodule formation in soybean and *L. japonicus,* species-
73 specific variation exists, such as the pseudoinfections in soybean (Calvert et al., 1984).

74 It has long been proposed that the auxin maximum in the founder cells of a nodule
75 primordium is controlled by polar auxin transport (Hirsch et al., 1989; Mathesius et al., 1998;
76 Kohlen et al., 2018); however, the underlying molecular mechanisms remain elusive. Ng and
77 Mathesius (2018) utilized *L. japonicus* and *M. truncatula* as model legumes to study the
78 differential regulation of auxin transport for initiating determinate and indeterminate nodules.
79 They proposed that acropetal auxin transport is not necessary for determinate nodule formation,
80 based on the observation that acropetal auxin transport in the equivalent root segments below
81 the rhizobia-inoculation spot decreases significantly in *M. truncatula,* while it increases in *L.*
82 *japonicus* (Pacios-Bras et al., 2003; Ng et al., 2015; Ng and Mathesius, 2018). This was further
83 supported the generation of a pseudonodule in *M. truncatula*, but not in *L. japonicus* upon
84 application of auxin transport inhibitors (Ng and Mathesius, 2018). Therefore, the mechanism
85 of determinate nodule formation is somehow different from that of the indeterminate type. So
86 far, much of our knowledge of the regulation of auxin for nodulation derives from the studies
87 of indeterminate nodule development and the knowledge gleaned from lateral root
88 organogenesis. In contrast, the functionality of auxin transport during determinate nodule
89 development remains speculative (Kohlen et al., 2018). Therefore, a better understanding of
90 the PIN-dependent auxin transport module for soybean nodule organogenesis is crucial for
91 discerning the basic nodulation mechanisms and uncovering the different regulatory
92 mechanisms between determinate and indeterminate nodule development.

93 In this study, we demonstrate that soybean nodule primordium formation involves
94 GmPIN1-mediated polar auxin transport. Among all canonical PINs, the GmPIN1 orthologs,
95 *GmPIN1b, c, d* were specially expressed in nodule primordium cells during nodule primordium
96 initiation, and their dynamic polarity indicate involvement in the directional auxin flux for
97 nodule primordium formation. We found that the upstream nodulation elicitors, flavonoids,
98 trigger the expanding distribution of stele-restricted *GmPIN1b* to cortical cells and cytokinin
99 enable the rapid redirection of the auxin stream by rearranging the cellular GmPIN1b polarity.
100 CRISPR/Cas9-based mutagenesis of *GmPIN1a, GmPIN1b* and *GmPIN1c* failed to establish a
101 focused auxin maximum in nodule meristem, resulting in aberrant cortical cell division.
102 Additionally, we found that the soybean-evolved *GmPIN9d* accumulates strongly in the

4

103   conjunctive vascular bundles between the root and nodule. In these tissues, GmPIN9d acts

104   synergistically with GmPIN1 to coordinate the auxin supply within the vasculature for nodule

105   enlargement. Our findings reveal the fundamental role of GmPIN-dependent polar auxin

106   transport for determinate nodule development.

107

108   **RESULTS**

109   **Auxin accumulates in the nodule primordium and vascular bundles**

110   To visualize the developmental process of soybean nodulation, 7-day-old soybean seedlings

111   were inoculated with the rhizobium strain *Bradyrhizobium* sp. (*BXYD3*). The root segments

112   below shoot-root junction area where large amounts of nodules accumulated (Supp. Fig. S1A)

113   were collected in series for further sectioning, at 4 days post-rhizobia inoculation (dpi) until 14

114   dpi.

115   On the basis of cell morphology, we divided nodule development into four stages. At

116   stage I, rhizobial infection via the curled root hairs determined the position of future nodule

117   primordia and subsequently resulted in cell proliferation in the outer cortex cells,

118   corresponding to nodule primordium initiation. At stage II, cortical cells underwent a series of

119   anticlinal and periclinal divisions to establish a multi-layered meristem. In particular, the

120   soybean nodule primordium started from the outer cortex layer and proceeded both outwards

121   and inwards, suggesting the presence of a dynamic regulator involved in modulating this dual

122   directional cell division. At stage III, the developing nodule continued to produce new cells,

123   and the protruding meristem contained a small amount of bacteroids and visible vascular

124   tissues that connected the host root and nodule. At maturity (stage IV), the oval nodule was

125   rapidly expanding and became filled with large amount of bacteroids, with complex vascular

126   bundles enveloping the entire nodule (Fig. 1A).

127   To track auxin distribution at different growth stages of soybean nodules, we generated

128   a stable transgenic soybean line with the synthetic *DR5-V2-GUS* auxin response reporter (Liao

129   et al., 2015) that shows higher sensitivity to endogenous auxin responses in Arabidopsis than

130   the original *DR5-GUS* reporter (Ulmasov et al., 1997). A robust auxin gradient with a peak

131   concentration at the tip of the nodule primordium, suggesting an early auxin response,

132   coincides with initial cell divisions of the nodule (Fig. 1B, stage I). When the outer cortex cells

133   were rapidly dividing and expanding, we found an accumulation of DR5-V2 activity with its

134   maximum at the apex of the nodule primordium (Fig. 1B, stage II). Afterwards, the tip-focused

135   auxin maximum disappeared and a new auxin maximum gradually appeared within the

136   surrounding cells near the root-nodule vascular connection (Fig. 1B, stage III). Within the

rapidly expanding stage IV nodule, auxin was concentrated throughout the nodule vascular bundles (Fig. 1B).

The auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) directly elicits the generation of nodule-like structures (pseudonodules) in legume species with indeterminate nodules (Hirsch et al., 1989; Wu et al., 1996; Rightmyer and Long, 2011), but not in those forming determinate nodules (Ng and Mathesius, 2018). We investigated whether NPA influences soybean nodule formation. Therefore, we incubated the rhizobium-colonized *DR5-V2-GUS* seedlings with NPA. NPA blocked auxin efflux and disrupted the auxin gradient, resulting in a strong ectopic DR5-V2 activity around the cortical cells (stage I), pericycle cells (stage II) and vascular bundles (stage III) (Fig. 1B). A reduced auxin response in the central zone of Stage III primordium was observed (Fig. 1B), suggesting a redirection of auxin transport occurs at this stage of nodule development. Interestingly, this was not seen in the NPA-treated plant nodules at the same stage. This ectopic NPA-driven auxin response activation was associated with onset of rapid cell divisions, resulted in a three-fold increase of small nodules compared to the mock treatment (Fig. 1A, Supp. Fig. S1B-D). Additionally, NPA disrupted vascular development, leading to aberrant nodules and two infection zones within bacteroids (Fig. 1A, stage IV). Collectively, these results suggest that nodule primordium formation requires the establishment of an appropriate auxin gradient and the further nodule development of the nodule vascular bundles involves a dynamic redirection of the auxin transport.

Identification of nodule-expressed *GmPINs*

The best-characterized regulators of auxin transport are PIN efflux transporters (Petrasek et al., 2006). The Arabidopsis PIN family consists of eight members, divided into canonical and non-canonical types based on the length of their hydrophilic loop and subcellular localization (Viaene et al., 2013). The canonical, PM-localized PINs play a predominant role in determining the directionality of intercellular auxin flow (Adamowski and Friml, 2015). To better understand the regulatory mechanism of auxin transport for nodule development, we were motivated to study the functionality of nodule-expressed canonical *GmPIN* ortholog. In soybean, the PIN family has extensively expanded because of the two whole-genome duplication events. A total of 23 *GmPINs* have been identified in the soybean genome, of which seven pairs of the duplicated genes, namely the *GmPIN1, GmPIN2, GmPIN3, GmPIN5, GmPIN6, GmPIN8* and *GmPIN9* subfamilies (Wang et al., 2015a). According to the constructed phylogenetic tree, the canonical *GmPIN* members comprise *GmPIN1 (GmPIN1a,*

171 *b, c, d, e), GmPIN2 (GmPIN2a, b)*, and *GmPIN3 (GmPIN3a, b, c, d)*, which showed the highest

172 sequence similarity with Arabidopsis *AtPIN1, AtPIN2,* and *AtPIN3, 4, 7,* respectively (Fig. 2A,

173 Supplemental File 1). Additionally, the soybean genome contains a specific *GmPIN9* group

174 that has apparently co-evolved with *GmPIN2* (Fig. 2A, Supplemental File 1&2).

175   To search for nodule-expressed *GmPINs*, we extracted RNA from nodules at 14 dpi to

176 perform reverse transcription quantitative PCR (RT-qPCR). Among the 23 *GmPINs*, the

177 canonical PIN orthologs, *GmPIN1*, *GmPIN2*, *GmPIN3* genes were detectable in the mature

178 nodule. Additionally, *GmPIN5a* and *GmPIN9d* transcripts were strikingly abundant in the

179 mature nodule, suggesting their possible involvement as well (Fig. 2B). The hydrophilic loop

180 of GmPIN9 is shorter than the canonical PINs but longer than that of the non-canonical type

181 (Supp. Fig. S2A); hence, the functionality of GmPIN9 during nodule development deserved

182 our further investigation.

183

184 **GmPIN1, orthologs of auxin efflux carrier AtPIN1, control directional auxin flow for**

185 **nodule primordium formation**

186 We first examined the expression profiles of canonical *GmPINs* during nodule development,

187 including *GmPIN1*, *GmPIN2* and *GmPIN3*. We established *pGmPIN1a-e:GUS, pGmPIN2a-*

188 *b:GUS* and *pGmPIN3a-d:GUS* constructs, using a reporter gene encoding β-glucuronidase

189 (GUS) individually driven by an approximately 2-kb region upstream of the *GmPIN* coding

190 sequence. We then introduced them into soybean seedlings via hairy root transformation.

191 Histochemical staining of the root tip revealed that every *GmPIN1* was expressed in the root

192 stele, consistent with the expression pattern of *AtPIN1* in Arabidopsis root (Supp. Fig. S3A).

193   Upon rhizobial infection, expression of the *GmPIN1b, c, d* reporters was detected in the

194 nascent nodule primordia that formed in outer cortical cells (Fig. 2C). After a series of cortical

195 cell divisions, the primordium tip-distributed *GmPIN1b, c, d* gradually accumulated in the

196 surrounding vascular bundles (Fig. 2C). In stage IV, the expression of *GmPIN1b, c, d* were

197 confined to vascular bundles (Fig. 2C). By contrast, *GmPIN1a* appeared after primordium

198 emergence and its expression was exclusively associated with vascular bundle development,

199 whereas *GmPIN1e* had an extremely weak signal in nodule primordia (Fig. 2C). *GmPIN1*

200 expression was associated with the nodule primordium and the nodule vascular bundle (Fig.

201 2D), consistent with the spatial pattern of auxin activity during nodule development. This

202 suggested the possible involvement of GmPIN1 in nodulation.

203   Other GmPIN members, *GmPIN2a* and *2b* were mainly expressed in root epidermis

204 and outer cortex cells, and *GmPIN3a-d* genes were expressed in root vascular cells (Supp. Fig.

205   S3B). By contrast, both *GmPIN2* and *GmPIN3* mainly accumulated in nodule vascular bundles

206   after establishment of the nodule primordium; only *GmPIN2a* and *GmPIN3a* were detectable

207   in the nodule primordium tip, albeit at extremely low levels (Supp. Fig. S3B). Our analysis of

208   the spatio-temporal expression pattern of canonical *GmPINs* lent further support for a

209   predominant role of GmPIN1 in nodule development.

210   The variation in the expression of multiple *GmPIN1* orthologs allowed us to evaluate

211   the functional conservation between GmPIN1 and AtPIN1. We established a GmPIN1-GFP

212   fusion protein under the control of the *AtPIN1* promoter. We cloned GFP in-frame in the

213   hydrophilic loop of GmPIN1 (Fig. 3A) at the comparable position, as in AtPIN1-GFP

214   (Benkova et al., 2003; Wisniewska et al., 2006) and introduced them into the Arabidopsis *pin1-*

215   *En* mutant. All the transgenic Arabidopsis *pAtPIN1:GmPIN1a-e-GFP* plants showed partial

216   rescue of the pin-shaped inflorescence meristem phenotype of the homozygous *pin1* mutant

217   (Galweiler et al., 1998), in that they had a high percentage of normal growing stems with intact

218   flowers and siliques (Fig. 3C). In the root stele, protein localization of GmPIN1a-e was

219   confined to the basal side of PM (Fig. 3B), consistent with the subcellular polarity of AtPIN1.

220   To further confirm the cellular polarity of GmPIN1a-e, we also generated transgenic

221   Arabidopsis plants having *GmPIN1a-e-GFP* driven by their native *GmPIN1a-e* promoters.

222   Similar to *pAtPIN1:GmPIN1-GFP* or in *pGmPIN1:GmPIN1-GFP* transgenic seedlings,

223   GmPIN1a-e were exclusively confined to the basal PM in root stele cells (Supp. Fig. S3C),

224   thus indicating the conservation of localization between GmPIN1 and AtPIN1 proteins.

225   On the basis of this functional conservation and consistent polarization of the five

226   GmPIN1 proteins, we next focused on GmPIN1b because it was detectable throughout all

227   stages of nodule development, using it as a representative to study the dynamics of GmPIN1

228   polarity during nodule development. For this, we generated a stable transgenic soybean line

229   transformed with *pGmPIN1b:GmPIN1b-GFP*. By comparison with wild-type (WT) plants, we

230   were able to distinguish the GFP signal from the strong auto-fluorescence of soybean tissues

231   under the same fluorescence microscopy settings (Supp. Fig. S4). We determined the degree

232   of polar localization of GmPIN1b as the signal ratio of apical relative to lateral side of

233   individual cells (called the polarity index, p.i.) (Fig. 3G). In the soybean root stele, GmPIN1b

234   displayed basal polarity (p.i.=2.6), consistent with its distribution in Arabidopsis (Fig. 3D, G).

235   During the nodule primordium initiation (stage I) with visible anticlinal cell divisions,

236   GmPIN1b showed predominant polarization towards the future nodule meristem (p.i.=3.2);

237   while some of the cells exhibited dual polarization of GmPIN1b, with PM accumulation

238   towards the imminently initiated nodule vascular bundle and towards the primary root tip (Fig.

239 3D, G). The predominant apical polarity of GmPIN1b towards the primordium apex suggests

240 that auxin is being transported to nodule primordia, while the bi-polar localization of GmPIN1b

241 implies that auxin is partly being directed away from the outer cortical cells.

242 In stage II, GmPIN1b polarity was mainly directed to the protruding nodule primordium

243 (p.i.=2.2); while a proportion of primordia cells displayed the lateral polarity of GmPIN1b,

244 which might result in reduced auxin response at the nodule primordium tip (Fig. 3D, 3G, Supp.

245 Fig. S5A). When the nodule primordium was fully established (stage III), GmPIN1b polarized

246 towards the primordium tip as well as in the lateral direction (p.i.=1.7) (Fig. 3D, 3G, Supp. Fig.

247 S5A), suggesting that GmPIN1 established a re-allocation route for draining auxin away from

248 the tip region. In the mature nodule (stage IV), the GmPIN1b-GFP signal was restricted within

249 vascular bundles (Fig. 3D, G), corresponding to the depletion of auxin at the primordium tip

250 and its reallocation to the vascular bundles (Fig. 1B).

251 We further examined whether GmPIN1b directs auxin flow toward the central cells of

252 the nodule primordium or away to the peripheral cells by evaluation of apical or lateral

253 GmPIN1b signal at stages I, II and III. Compared to the GmPIN1b signal in stage I, the apical

254 direction of the GmPIN1b signal was significantly reduced to 53% and 62% in stage II and

255 stage III+IV, while the lateral GmPIN1b signal was not promoted in the later stages (Fig. 3F).

256 The comparison of GmPIN1b polarization at different stages of nodule development further

257 indicated that the re-allocation of auxin away from nodule primordium tip is caused by the

258 reduction of apical polarization of GmPIN1.

259 In vascular bundles of mature nodules, GmPIN1b was highly polarized within the

260 differentiated vasculature (p.i.=21.0) but was non-polar within the undifferentiated vasculature

261 (p.i.=1.0, Fig. 3D, G). The polarization of GmPIN1b within the mature vasculature of root-

262 nodule junction suggested that auxin might be directionally transported via the nodule

263 vasculature. Interestingly, GmPIN1b polarity was disrupted by NPA. NPA relocated the

264 GmPIN1b polarity to the lateral side that was perpendicular to nodule primordium cells

265 (p.i.=1.4 in NPA-treated nodule primordia, compared to p.i.=2.3 in the untreated ones) (Fig.

266 3E-G, Supp. Fig. S5B). Consequently, NPA-rearranged GmPIN1 polarity resulted in ectopic

267 formation of nodule primordia (Fig. 1A, 3E). Taken together, the dynamic polarization of

268 GmPIN1 in the nodule primordium is consistent with the presence of multiple cell proliferation

269 sites during soybean determinate nodule development, which begins in the outer cortex and

270 proceeds outwards as well as inwards.

271

272 **GmPIN1s regulate nodule primordium formation**

Soybean has evolved five *PIN1* orthologues compared with the sole *PIN1* identified in Arabidopsis, suggesting a functional redundancy of *GmPIN1* genes. Among them, *GmPIN1a, b* and *c* showed the closest sequence similarity to *AtPIN1* (Fig. 2A), while *GmPIN1d* and *e* might be an ancestral PIN1 form (Kohlen et al., 2018). Using the CRISPR/Cas9 approach, we designed a sgRNA that simultaneously edits the first exon of *GmPIN1a, b* and *c*, from which, we subsequently obtained two independent, triple mutants of *Gmpin1abc* (named *Gmpin1abc-*L1 and -L2) via *Agrobacterium*-mediated stable transformation in soybean (Fig. 4A). These *Gmpin1abc* mutants displayed slightly shorter primary roots, fewer lateral roots, and had less biomass in their shoot tissues, compared to WT (Supp. Fig. S6A-D).

To understand the contribution of GmPIN1 to auxin transport, we examined the auxin transport capacity in *Gmpin1abc* mutants. Without rhizobium inoculation, *Gmpin1abc* mutants' acropetal auxin transport was reduced by 89%, compared to WT (Fig. 5H). The decreased acropetal auxin transport in *Gmpin1abc* mutants resulted in strongly reduced auxin content (46%-53%) compared to WT, and a reduced *DR5-V2*-GUS activity (Supp. Fig. S6E, F). Quantification of the total nodule number (per plant) and nodule density (nodule number per cm^2 of root area) both demonstrated that *Gmpin1abc* had fewer nodules than the WT (Fig. 4B, C, E). Despite the reduction in nodulation events in *Gmpin1abc* mutants, their average nodule size was not significantly changed, as indicated by diameter distribution profile of more than 1000 nodules (Fig. 4D, F). The nodulation phenotype of the *Gmpin1abc* mutant indicates that GmPIN1 is involved in regulation of nodule formation.

We then generated stable transgenic plants that constitutively overexpressed *GmPIN1a-c* (*35s:GmPIN1a-c*). The *35s:GmPIN1a-c* lines all exhibited curling roots and an unusual thickening of hypocotyls (Supp. Fig. S7A, B), features similar but not entirely identical to agravitropic root growth seen in Arabidopsis *35s:AtPIN1* plants (Zhang et al., 2010). Further analysis of nodulation phenotypes showed that the induction of *GmPIN1a, b* and *c* all significantly reduced nodule size (Fig. 4G-J). The retarded growth of *35s:GmPIN1* nodules could be due to the disrupted auxin maximum at nodule primordia, leading to delayed organogenesis, or due to an impaired auxin concentration within the enlarged nodule that impeded nodule expansion.

To understand the causal relationship of the defective nodulation phenotype in *Gmpin1abc* mutants and gain-of-function *GmPIN1* lines, we examined the density of nodule primordia at 5 dpi, when most of the nodules had just emerged as primordia. We divided the nodules into two categories: primordia (without the resident bacteroids) and developing nodules (with bacteroids). Compared with WT, *Gmpin1abc* mutants had a significantly

307   decreased density of nodule primordia but this did not influence their developing nodules (Fig.

308   5A-C), further supporting the involvement of GmPIN1 in nodule primordium formation.

309   Strikingly, *35s:GmPIN1a* has almost no detectable nodule primordia at 5 dpi (Fig. 5A-C, I).

310   To further understand the functionality of GmPIN1-mediated auxin transport during

311   nodule primordium formation, we established a spot inoculation system in soybean (Fig. 5D)

312   to closely examine auxin transport efficiency and nodule primordium morphology within the

313   spot inoculation area. To this end, 0.5-cm root segments of WT and *Gmpin1abc* mutants below

314   the root-shoot junction were subjected to a continuous *BXYD3* spot inoculation for 48h (Fig.

315   5D, Supp. Fig. S7C). Auxin concentration was individually measured in the above (upper, U)

316   and below (lower, L) root segments close to the inoculated site. In WT, rhizobial infection did

317   not influence auxin levels in upper root segments, but decreased the auxin content to 72% in

318   the lower segments, compared to the uninfected roots (Fig. 5G). Auxin concentration was

319   generally decreased in *Gmpin1abc* roots, compared to WT. In *Gmpin1abc* mutants, rhizobial

320   inoculation did not change auxin level in the upper root segments but caused auxin elevation

321   in the lower segments (247% and 196%) (Fig. 5G).

322   The opposite responsiveness of WT and *Gmpin1abc* intrigued us to further examine

323   auxin transport rates of inoculated root segments. Radiolabeled [^{3}H]-IAA was applied to the

324   upper segments and the transported [^{3}H]-IAA was measured in the lower segments at 48 hours

325   post inoculation (hpi). In WT or *Gmpin1abc-L2* mutants, rhizobial inoculation did not

326   significantly change the efficiency of acropetal auxin transport across the inoculated roots (Fig.

327   5H). It is worth noting that rhizobial inoculation tended to decrease auxin transport in WT

328   (92%), while it tended to increase auxin transport in *Gmpin1abc-L1* and *L2* mutants (121% and

329   111%), compared to the uninfected samples (Fig. 5H). Reduction of auxin levels below the

330   inoculation site supported the notion that auxin transport regulation happens prior to the

331   formation of visible nodule primordia. Whereas, the minor change of acropetal auxin transport

332   upon rhizobium infection suggested that nodule primordium initiation in the outer cortex is not

333   sufficient to influence acropetal auxin transport within the primary root vasculature. The

334   unexpected elevation of auxin level in lower segments of *Gmpin1abc* suggests that a possible

335   upregulation of auxin biosynthesis occurs within inoculated-*Gmpin1abc* roots.

336   We further examined nodule primordium morphology at 5 days after rhizobia spot-

337   inoculation. The visible nodule primordium with a series of anticlinal and periclinal divisions

338   were homogenously distributed in inoculated WT root segments, while clusters of aberrantly

339   dividing cortical cells and nodule primordia were discretely distributed in *Gmpin1abc* mutants

340   (Fig. 5E), compared to the normal cortical cells in the uninfected roots (Supp. Fig. S7D). We

then introduced *DR5-V2-GUS* in WT, *Gmpin1abc* mutants and *35s:GmPIN1a* to visualize the auxin distribution. A robust auxin gradient with a peak concentration was present at the tip of WT nodule primordium (stage I) (Fig. 5F). Despite the *DR5-V2* signal remaining clear in nodule primordium of *Gmpin1abc* mutant, the mutant failed to establish a focused auxin gradient in the primordium apex (stage I) (Fig. 5F). The defective auxin maximum in *Gmpin1abc* resulted in the ectopic formation of nodule primordia, leading to the clustered spacing of nodule primordia. By contrast, overexpression of *GmPIN1a* in *35s:GmPIN1a* roots resulted in ectopic auxin deposition in the cells beneath the primordium tip, which interfered with the directional auxin transport that would guide nodule primordia to grow outwards. Consequently, *35s:GmPIN1a* exhibited a delay in nodule organogenesis (Fig. 5A, F). These results collectively demonstrate that a GmPIN1-mediated auxin gradient regulates nodule primordium formation.

Flavonoids and cytokinin influence PIN1 distribution and polar localization during nodulation

An auxin maximum and *GmPIN1b, c, d* expression emerged in nodule primordium founder cells, suggesting the involvement of auxin transport in the initial step of nodule formation. However, *GmPIN1a, b, c* expression was exclusively restricted within the root vasculature (Supp. Fig. S3A), which pointed to an unknown nodulation signal eliciting the lateralization of GmPIN1 to prime the auxin signal at these founder cells.

Flavonoids are crucial signaling molecules that can trigger the synthesis of Nod factors from rhizobia, ensuring successful symbiotic infection of host plant tissues (Liu and Murray, 2016). A variety of flavonoid compounds have been characterized, and most of them act as inhibitors of auxin transport (Brown et al., 2001; Santelia et al., 2008; Ng et al., 2015) by interfering with PIN expression and its polar localization (Peer et al., 2004; Santelia et al., 2008; Kuhn et al., 2017). According to the multiple steps of signal exchange during nodule formation, different flavonoids may play distinct roles across individual steps of nodulation (Zhang et al., 2009). The isoflavone compound genistein is a well-characterized Nod-factor inducer in soybean (Ip et al., 2001; Subramanian et al., 2006; Lang et al., 2008). We thus checked the effect of genistein on the expression pattern of *GmPIN1b*, serving as a representative *GmPIN1*. Soybean plants with *pGmPIN1b:GUS* showed an expanded distribution of *GmPIN1b* into their cortical cells by genistein treatment (Supp. Fig. S8A, C). The flavonoid 7,4'-dihydroxyflavone (DHF) is a Nod gene-inducing flavone compound in *M. truncatula*, known to function very early during nodulation (Zhang et al., 2009). Application of exogenous DHF to soybean

12

375 *pGmPIN1b:GUS* trans-genetic plants also showed an expanded distribution of *GmPIN1b* into

376 the cortical cells (Supp. Fig. S8A, C), consistent with the genistein treatment. These results

377 indicated that the isoflavone genistein and flavonoid DHF affect GmPIN1 distribution.

378 Corresponding to the expanded distribution of GmPIN1b, genistein and DHF treatment

379 resulted in a significantly increased *DR5-V2-GUS* signal along the outer cortex and epidermal

380 cells, but a decrease in quiescent center of root tip (Supp. Fig. S8B, D). These data collectively

381 demonstrate that the isoflavone/flavonoid influences GmPIN1 distribution and auxin

382 accumulation, and probably leading to the alteration of auxin transport during nodulation.

383 Appropriate integration of various phytohormones is essential for determining when

384 and where organogenesis occurs, such as the coordination between auxin and cytokinin

385 (Schaller et al., 2015; Pierre-Jerome et al., 2018; Kurepa et al., 2019). Cytokinin plays a major

386 role in the control of cortical cell divisions during nodule primordium formation (Murray et al.,

387 2007; Reid et al., 2017), which consequently promotes nodule numbers (Supp. Fig. S9A, B).

388 To understand whether cytokinin functions upstream of auxin, we applied a synthetic cytokinin,

389 6-benzylaminopurine (6-BA), onto *pGmPIN1b:GmPIN1b-GFP*. Visualization of GmPIN1b

390 localization revealed that 6-BA rearranged the cellular GmPIN1b polarity that predominantly

391 targeted to primordium tip, resulting in a switch of GmPIN1b polarity to the lateral side that

392 was perpendicular to the primordium cells (Fig. 6A, B). Cytokinin-mediated directional

393 alteration of PIN polarity during nodule meristem formation was consistent with its effect on

394 lateral root initiation (Marhavy et al., 2014). Interestingly, a directional alteration of GmPIN1

395 polarity by 6-BA treatment resulted in a series proliferation in the outer cortical cells of WT

396 plants, comparable with the cell morphology in spot inoculated-*Gmpin1abc* mutants (Fig. 6C,

397 5G). Hence, these findings suggested that cytokinin-regulated re-polarization action of

398 GmPIN1 might facilitate a rapid auxin stream redirection, which provides an efficient approach

399 to initiate nodule primordium. Taken together, the influence of flavonoid and cytokinin on

400 GmPIN1 cellular localization might coordinate an appropriate auxin gradient for nodule

401 organogenesis.

402

403 **GmPIN9d is a nodule vascular bundle-abundant auxin transporter**

404 The above results confirmed the important role of GmPIN1 during nodule primordium

405 formation. Apart from the typical canonical and non-canonical GmPINs, there was an

406 additional GmPIN9 subfamily conspicuously present in soybean that showed a very close

407 evolutionary relationship with the PIN2 clade (Fig. 2A). Our RT-qPCR analysis of *GmPIN9a-*

408 *d* expression levels showed that *GmPIN9d* was extremely enriched in the mature nodule (Fig.

13

409 7C). In contrast, the transcripts of *GmPIN9a*, *GmPIN9b*, *GmPIN9c* were rather low or not

410 detectable (Fig. 7C), making GmPIN9d the most likely candidate involved in nodulation.

411   We next generated a stable transgenic soybean plant with a GUS reporter gene driven

412 by the 2-kb promoter region of *GmPIN9d*. A series of cross-sections spanning leaf to root

413 tissues, integrated with histochemical staining, from 10-day-old *pGmPIN9d:GUS* seedlings

414 demonstrated that *GmPIN9d* was weakly expressed in aerial tissues but enriched in both root

415 and nodule. Either in the hypocotyl or root organs, *GmPIN9d* was confined within the vascular

416 bundles (Fig. 7A, B). Histochemical co-staining of xylem cells using phloroglucinol confirmed

417 that *GmPIN9d* was located in protoxylem cells (Fig. 7A). Intriguingly, *GmPIN9d* was

418 particularly abundant in the conjunctive vascular bundles lying between the root and nodule

419 (Fig. 7B), which suggested a potential role of GmPIN9d in the later stage of nodule growth

420 when primordia were already established.

421   Because GmPIN9d is a soybean-evolved PIN, we tested if GmPIN9d is indeed a

422 functional auxin transporter. To do this, we cloned GmPIN9d-GFP under control of the

423 Arabidopsis PIN2 promoter (*pAtPIN2:GmPIN9d-GFP*) and introduced it in the Arabidopsis

424 PIN2 knockout mutant, *eir1-4* that showed agravitropic roots (Muller et al., 1998). Compared

425 to *pAtPIN2:GmPIN2b-GFP* which entirely rescued the agravitropic root phenotype of *eir1-4*

426 mutant, *pAtPIN2:GmPIN9d-GFP* displayed a partial restoration (Supp. Fig. S11A-C),

427 suggesting that GmPIN9d might carry out a comparable function as PIN2 protein for polar

428 auxin transport. Importantly, much of the GmPIN9d-GFP localized to the internal structures

429 reminiscent of the endoplasmic reticulum (ER) (Supp. Fig. S11B), suggesting that it either acts

430 at the ER, or that its trafficking to the membrane is impaired in Arabidopsis.

431   We further generated *Gmpin9d* mutants using CRISPR/Cas9. Two homozygote mutant

432 alleles were identified, designated *Gmpin9d-#1* and *9d-#2*, with an early stop codon that arose

433 from a 2-bp and 40-bp deletion, respectively (Fig. 8A). The 7-day-old *Gmpin9d* mutants had

434 slightly decreased length and density of lateral roots, but did not display other obvious

435 developmental defects in adult plants (Fig. 8B, Supp. Fig. S10A-C). Next, the auxin response

436 reporter *DR5-V2-GUS* was introduced to hairy roots of the WT and the *Gmpin9d* mutant. The

437 *DR5-V2-GUS* activity was much reduced in both the roots and nodules of *Gmpin9d* mutant

438 (Fig. 8C), supporting the involvement of GmPIN9d in auxin homeostasis.

439   The GmPIN9 clade is located between canonical and non-canonical PIN types,

440 according to our phylogenetic analysis (Fig. 2A). This raised a problem: might GmPIN9d

441 polarly transport auxin as a canonical PIN, or does it regulate auxin homeostasis as a non-

442 canonical PIN? To address these questions, we measured free and conjugated auxin levels in

14

both the WT and *Gmpin9d* mutants, using High-Performance Liquid Chromatography (HPLC). In comparison to WT, the free auxin level decreased by approximately 50% in roots and 20% in nodules of *Gmpin9d* mutants (Fig. 8G-H), results consistent with the previous observation of *DR5-V2* activity (Fig. 8C). However, the conjugated form of auxin, indole-3-acetyl-aspartate (IAA-asp), in *Gmpin9d* mutants occurred at a level comparable to WT (Supp. Fig. S10D-E), supporting a transporter function of GmPIN9d. Finally, using radiolabeled [^{3}H]-IAA, we measured the auxin transport capacity of GmPIN9d in 2-cm equivalent root segments of the WT and *Gmpin9d* mutants. Both *Gmpin9d-#1* and *#2* mutants underwent a significant decrease in auxin transport in the rootward (acropetal) but not shootward (basipetal) direction, in comparison with WT (Fig. 8D-F). Altogether, these results show that GmPIN9d can transport auxin to the root tip.

GmPIN9d-dependent auxin transport is required for nodule enlargement

Although the free auxin level was diminished by approximately 50% in roots of *Gmpin9d* compared to WT, the density of nodules and nodule primordia were not significantly changed (Fig. 8I-J). However, the profile of nodule size in *Gmpin9d* was slightly shifted compared to WT (Fig. 8K), suggesting the involvement of GmPIN9d for nodule expansion. Still, the phenotypic defects of *Gmpin9d* single mutants were mild might be caused by complicated gene redundancy of PIN family. The acropetal auxin transport capacity of GmPIN9d was reminiscent of GmPIN1, prompting us to speculate that GmPIN9d might work synergistically with GmPIN1 to achieve polar auxin transport within nodule vascular bundles.

We then examined the subcellular localization of GmPIN9d, via visualization of the Arabidopsis transgenic *pAtPIN2:GmPIN9d-GFP* seedlings. GmPIN9d showed dual localization in both ER and PM by colocalization of GmPIN9d-GFP with FM4-64-stained PM and HDEL-labelled ER (Supp. Fig. S11D-E). PM-associated PIN proteins are located on the PM, meanwhile be trafficking among endomembrane system via endocytosis and exocytosis pathways (Chen et al., 2011; Adamowski and Friml, 2015).

To further investigate whether GmPIN9d is associated with the PM, we applied Brefeldin A (BFA), which is widely used as a vesicle trafficking inhibitor (Geldner et al., 2003). BFA specifically blocks exocytosis but allows endocytosis, aggregating both secretory and endocytic endomembrane cargoes in BFA bodies. BFA treatment specifically aggregated the PM-localized GmPIN2b, but not the ER-resident AtPIN8 in BFA bodies (Supp. Fig. S11F). We found that BFA also resulted in the aggregation of GmPIN9d proteins within BFA

476 compartments (Supp. Fig. S11F). These data in Arabidopsis clearly evinced a dual localization

477 of GmPIN9d in both PM and ER, similar to AtPIN6.

478   We further detected the *in vivo* distribution of GmPIN9d in soybean roots and nodules,

479 by placing the GmPIN9d-GFP fusion protein under the control of the native GmPIN9d

480 promoter (Fig. 9A). The transformed positive hairy roots were selected based on a dTomato

481 fluorescence-based screening system (Supp. Fig. S12A). In *pGmPIN9d:GmPIN9d-GFP*

482 positive hairy roots, a strong GFP signal was present in the root protoxylem, with rootward PM

483 localization (Fig. 9B). In particular, this GmPIN9d-GFP signal was associated with vascular

484 bundles within the nodule and root-nodule conjunction. The strong polarization of GmPIN9d

485 within root-nodule conjunctive vasculature suggested its possible involvement in directing

486 auxin stream towards nodule (Fig. 9B, Supp. Fig. S11G). Such a GmPIN9d polarized pattern

487 resembles GmPIN1 at a later stage of nodule development, pointing to their synergistic activity.

488 Hence, we introduced the *Gmpin9d-RNAi* construct in *Gmpin1abc* triple mutants via the

489 dTomato-based hairy root screening system. The resulting *Gmpin1abc Gmpin9d-RNAi* showed

490 a comparable nodule density but much smaller nodule size than *Gmpin1abc* (Fig. 9C-E, Supp.

491 Fig. S12B-D). Taken together, the above results demonstrated that GmPIN9d-dependent polar

492 auxin transport functions at the later stage of nodule development, collaborating with GmPIN1

493 to fine-tune nodule enlargement.

DISCUSSION

Legume plants interact with rhizobia to generate nodule organs, representing a way by which legume species coordinate the mutually beneficial exchange of signals and nutrients with their symbiotic partners. Previous studies have characterized well the crucial roles of Nod factor recognition, nodule inception (NIN) transcription factor, and phytohormone cytokinin in nodule formation (Ng et al., 2015; Schiessl et al., 2019; Bozsoki et al., 2020; Laffont et al., 2020). Here, we showed that dynamic GmPIN1 polarization establishes auxin canalization during nodule primordium development; flavonoids influence GmPIN1 distribution and cytokinin rearranges the cellular GmPIN1 polarity, participating in auxin gradient formation. The later stage of nodule enlargement involves the collaboration of GmPIN9d and GmPIN1-dependent auxin transport within nodule vasculature (Fig. 9F). Our data thus provide insights into auxin-regulated nodule development in legume plants.

Establishment of an auxin maximum is required for determinate nodule primordium formation

Auxin accumulation appears in the early stage during nodule primordium formation, when the rhizobia-elicited infection threads start to grow in the proliferating cortical cells of *L. japonicus* (Suzaki et al., 2012), suggesting that nodule primordium formation is tightly accompanied by an auxin response. In soybean, overexpression of a nodule-expressed auxin biosynthesis enzyme *GmYUC2a* increases auxin content by approximately two fold, but severely delays nodule formation (Wang et al., 2019). These data suggests that maintenance of appropriate auxin concentration and distribution is a prerequisite for determinate nodule primordium formation. If auxin transport is not required for determinate nodule primordium formation, there should be a burst of local auxin biosynthesis at the nodule primordium site. YUCCA flavin monooxygenases are the major enzymes catalyzing IAA synthesis (Mashiguchi et al., 2011; Stepanova et al., 2011; Zhao, 2018). In soybean, *GmYUC2a* is the most abundant nodule-expressed YUC among all GmYUCs (Wang et al., 2019); however, *GmYUC2a* displays uniformly high activity in all participating cell layers during nodulation, including the root hairs, proliferating cortex cells, and nitrogen fixation zone (Wang et al., 2019). It is unlikely that a localized auxin maximum, restricted to nodule primordium founder cells, can be established solely relying on GmYUC2a. PIN-mediated polar auxin transport has been well characterized and deemed necessary for almost all organogenesis processes in plants. This raises the hypothesis that PIN-mediated auxin transport could also contribute to formation of determinate nodules.

17

528   Subramanian and colleagues mentioned that no measurable inhibition of auxin

529   transport occurred at the site of rhizobial inoculation where flavonoids/isoflavonoids

530   accumulate in soybean root, and they concluded that the effects of flavonoids on auxin

531   redistribution is not required for soybean nodulation (Subramanian et al., 2007). Our study

532   confirmed that acropetal auxin transport is not significantly affected in the rhizobia-inoculated

533   root segments (Fig. 5H). Different kinds of flavonoids are synthesized at distinct stages of

534   nodule formation (Mathesius et al., 1998), and the fluorescent flavonoids were visualized in

535   different cell layers of distinct legumes. For instance, flavonoids were found in the inner

536   cortical cells of pea (*Pisum sativum*) and alfalfa (*Medicago sativa*), but they were found in the

537   outer cortical cells of siratro (*Macroptilium atropurpureum*) (Mathesius et al., 1998). These

538   data suggest that different flavonoids play diverse roles during different stages of nodule

539   formation and in different species of legume plants.

540   Flavonoids have a general inhibitory effect on auxin transport (Buer et al., 2013; Ng et

541   al., 2015; Zhang et al., 2021), and acropetal auxin transport was disrupted during indeterminate

542   nodule formation where high amounts of flavonoids were synthesized. However, soybean

543   nodules initiate in the cells of outer cortex, and flavonoid compounds are probably also

544   synthesized at the outer cortex of soybean roots, lying far from the phloem. This could explain

545   why acropetal auxin transport is not affected during nodule primordium formation in soybean.

546   To understand whether flavonoid-mediated auxin inhibition is a necessary step for nodule

547   primordium initiation in soybean, it is necessary to closely examine GmPIN1 distribution and

548   auxin gradient in weak alleles of isoflavone synthase (IFS) or chalcone synthase (CHS)

549   mutagenized soybean plants within nodule primordium cells. Despite flavonoids being the best

550   candidates to prime the auxin signal, an obvious question remains unresolved. Soybean nodule

551   are initiated at the outer cortex cells, while *L. japonicus* nodule are initiated at the inner cortex

552   cells and the *M. truncatula* nodule begins from the pericycle, endodermis and cortex cells

553   (Gauthier-Coles et al., 2018). So, how does the deposition of flavonoids in different cell layers

554   in different kinds of legume plants modulate PINs and auxin transport?

555   In the present study, we demonstrate that a PIN-dependent auxin transport module

556   participates in soybean nodule primordium formation. *GmPIN1b*, *c, d* target to the founder

557   cells of nodule primordia, reallocating the auxin stream towards the nodule primordium and

558   flows away from the primordium. A proposed mathematic model postulates that nodule

559   formation is associated with the reduction of PIN function and an increase of auxin

560   concentration in all root cell layers (Xiao et al., 2014). Nevertheless, this auxin-involved

561   nodulation module is based on specific auxin accumulation from the inner to outer cell layers;

562   hence, the proliferation of cortex cells should start from the interior layers and proceed

563   outwards (Xiao et al., 2014). Yet, the determinate nodule exhibits dual directions of cell

564   proliferation, in that it begins from cortex cells and proceeds outwards as well as inwards.

565   Therefore, only the initial steps of nodule primordium development (proceed outwards) fit with

566   the proposed model (Xiao et al., 2014).

567   NPA disrupts auxin gradient formation during nodule primordium formation. In stage

568   I, auxin accumulates with its maximum at the nodule primordium apex, compared with a bulk

569   deposition of auxin along the dividing cortical cells in NPA-treated group; in stage II and III,

570   auxin response gradually reduces from the nodule primordium apex, compared with an ectopic

571   auxin accumulation in and around the dividing cortical cells (Fig. 1B). Together, the three-fold

572   increase of small nodules in NPA-treated roots indicates that disruption of auxin efflux by NPA

573   causes ectopic nodule primordium formation. Similar to the NPA treatment, loss of

574   *GmPIN1ABC* impairs polar auxin transport, generating clusters of abnormal dividing cells and

575   defective nodule primordia after continuous rhizobial inoculation (Fig. 5E). Hence,

576   establishment of an appropriate auxin gradient is a prerequisite to control nodule primordia

577   positioning. Whereas, nodule number was promoted in NPA-treated roots, which was opposite

578   with the consequence of decreased nodules in *Gmpin1abc* mutants. NPA directly associates

579   with PINs, and it inhibits PINs activity by stabilizing PIN1, PIN2, PIN3, PIN4 and PIN7

580   homo- and heteromers in Arabidopsis (Abas et al., 2021; Teale et al., 2021). Apparently, NPA

581   not only inhibits GmPIN1 activity, but also functions on other GmPINs. Hence, NPA-induced

582   nodules might be caused by the additive effects of NPA on all GmPIN proteins.

583

584   **Auxin supply is involved in nodule enlargement**

585   Despite acropetal auxin transport capacity and auxin concentration being severely decreased

586   in the root and nodule of the *Gmpin9d* single mutant, it did not display significant defects in

587   nodulation. A plausible explanation for this is that GmPIN9d functions at later stages of

588   nodulation where it is highly redundant with other PINs, including GmPIN1, GmPIN2 and

589   GmPIN3. Mutagenesis of multiple *GmPINs* recently became possible via CRISPR/Cas9. After

590   nodule primordia are formed, auxin maxima are rapidly concentrated within nodule vascular

591   bundles, suggesting an additive effect of other auxin transporters for auxin canalization

592   between the primordium and vascular bundles. Therefore, the nodule-expressed *GmPIN2* and

593   *GmPIN3* might also participate in later stages of nodule development. The fundamental effect

594   of auxin has been well known to stimulate cell expansion (Du et al., 2020). Hence, auxin stream

595 in vascular bundles could be a source of auxin to maintain auxin supply within the developing

596 nodule, modulating nodule enlargement.

597

598 **MATERIALS AND METHODS**

599

600

601 **Plant growth and phenotype analysis**

602 Soybean (*Glycine max*) cultivar Williams 82 and Huachun6, and *Arabidopsis thaliana*

603 *Columbia-0* were used as wild type (WT). Seeds of *Arabidopsis thaliana* were sown on 0.8%

604 agar containing 1/2 Murashige and Skoog media at 22°C under 16h light/ 8h dark photoperiod.

605 Soybean seeds were germinated in vermiculite. To analyze nodulation phenotype, 7-day-old

606 plants were inoculated with *Bradyrhizobium sp.* (*BXYD3*) which was re-suspended in low-

607 nitrogen buffer (530 µM N, including KNO_3, NH_4NO_3, $Ca(NO_3)_2 \cdot 4H_2O$, and

608 $(NH_4)_2SO_4$(15:4:12:3), and 1.2 mM $CaCl_2$, 1.05 mM K_2SO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 25 µM

609 $MgCl_2$, 2.5 µM $NaB_4O_7 \cdot 10H_2O$, 0.5 µM $MnSO_4 \cdot H_2O$, 1.5 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM

610 $CuSO_4 \cdot 5H_2O$, 0.15 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 40 µM Fe-Na-EDTA, 250 µM KH_2PO_4) to

611 OD600=0.15 for 2 h, then these inoculated plants were transferred to soil (vermiculite, and

612 irrigated with low-nitrogen buffer) for an additional 14 days (25°C, 14h light/ 10h dark

613 photoperiod). Light growth condition for soybean plants is white light added with blue and red

614 LEDs, the light intensity is 13000 LUX (measured by an HR-350 Light Meter; Hipoint). The

615 resultant plants were collected for nodule number and density quantification by ImageJ.

616　　　For NPA treatment, soybean seeds were germinated in vermiculite. 7-day-old plants

617 were inoculated with *BXYD3* (OD600≅1.0) which was re-suspended in low-nitrogen buffer

618 for 2 h, then the inoculated plants were transferred to low-nitrogen hydroponic solution with

619 or without 1-N-naphthylphthalamic acid (NPA) (10^{-7} M) for additional 21 days. Nodules were

620 harvested and analyzed.

621　　　For DHF, genistein or 6-BA chemical treatments, DHF and genistein were dissolved in

622 DMSO, and 6-BA was dissolved in NaOH as stock solutions. 7-day-old seedlings were

623 irrigated by chemical-containing low-nitrogen solution.

624

625 **RNA extraction and RT-qPCR analysis**

626 RNA was extracted using the TransZol Up Plus RNA kit (TransGen Biotech), and the first-

627 strand cDNA was synthesized using TransScript All-in-One SuperMix (TransGen Biotech).

628 qRT-PCR was performed using a Bio-Rad CFX96 real-time system (Bio-Rad) with 20 µL

629   volumes containing 2 µL of 1:5 diluted reverse transcription product, 0.8 µL of specific primers,

630   7.2 µL of ddH$_2$O, and 10 µL of 2×TransStart Green qPCR SuperMix (TransGen Biotech). The

631   housekeeping gene *GmActin-11* was used as a reference and relative expression levels of each

632   gene were calculated using the $2^{-\Delta\Delta CT}$ method. All primers used in this study are listed in

633   Supplemental Table S1.

634

Phylogenetics and motif analysis

636   *Arabidopsis thaliana PIN* genes were obtained from TAIR (http://www.arabidopsis.org).

637   Soybean *PIN* genes were obtained from Phytozome (https://phytozome.jgi.doe.gov).

638   *Klebsormidium flaccidum PIN* (*KfPIN*) was obtained from Genbank

639   (https://www.ncbi.nlm.nih.gov). *PIN* genes were translated into protein sequences and aligned

640   with Clustal X (Larkin et al., 2007). Phylogenetic analysis was constructed using MEGA X

641   with neighbor-joining (NJ) criteria and verified using the maximum likelihood (ML) method

642   (Kumar et al., 2018), and 1000 bootstrap replicates were performed based on the multiple

643   alignments of the protein sequences encoded by *PIN* genes. NJ analysis was performed using

644   the protein Poisson distances and the pairwise deletion of gap sites. The default parameters

645   were used for ML analysis. Moreover, Phylogenetics conserved motifs were detected in PIN

646   members using the motif analysis tool MEME (http://meme-suite.org/tools/meme) (Bailey et

647   al., 2009) with the default parameters except for maximum number of motifs, 25. The PIN

648   sequence alignment is provided as Supplemental File S1 and a machine-readable alignment is

649   provided as Supplemental File S2.

650

Used primers, vectors and cloning strategy

652   Promoter fragments of *GmPIN9d* (2003bp), *GmPIN1a* (2132bp), *GmPIN1b* (2043bp)

653   *GmPIN1c* (1917bp) *GmPIN1d* (1809bp) and *GmPIN1e* (1476bp) were amplified from

654   Williams 82 genomic DNA. The fragments were cloned into pDONR221 using Gateway BP

655   Clonase II enzyme mix and recombined into the pGWB633 Gateway destination vector by LR

656   reaction to create *pGmPIN:GUS* (primers are provided in Supplemental Table S1). To create

657   *pAtPIN1:GmPIN1a-e-GFP, pGmPIN1a-e:GmPIN1a-e-GFP, pGmPIN9d:GmPIN9d-GFP,*

658   *pAtPIN2:GmPIN2b-GFP* and *pAtPIN2:GmPIN9d-GFP*, the insertion site of GFP was

659   described in previous studies (Benkova et al., 2003; Xu and Scheres, 2005; Wisniewska et al.,

660   2006). GFP was inserted into the genomic fragments at position 1646 (after ATG) in *GmPIN9d*,

661   at position 1680 in *GmPIN2b*, at position 1396 in *GmPIN1a*, at position 1547 in *GmPIN1b*, at

662 position 1407 in *GmPIN1c*, at position 1914 in *GmPIN1d* and at position 1904 in *GmPIN1e*.
663 Genomic DNA of *GmPINs* and *GFP* were cloned into pBGWK by infusion clone technology.
664   To generate *Gmpin1abc* triple mutant and *GmPIN9d* single mutant, sgRNA was
665 designed according to the prediction from CRISPR-GE (http://skl.scau.edu.cn/home/). sgRNA
666 cloning approach was performed as described previously (Bai et al., 2020). To generate the
667 constructs with modified dTomato-tag, dTomato fragment was amplified using the primers of
668 pGmEF1A-dTomato F2 and R2, which was driven by the promoter of pGmEF1a; Tnos
669 fragment was amplified using the primers of pGmEF1A-dTomato F3 and R3. Then, pGmEF1a,
670 dTomato and Tnos were assembled by Goldengate assembly kit using BsaI and T4 ligase.
671 Finally, pGmEF1a-dTomato fragments were assembled with other fragments to create
672 destination vectors via Goldengate cloning methods. All strategies of vectors and cloning used
673 in this study are listed in Supplemental Table S2. Locus numbers of *GmPIN* genes are listed in
674 Supplemental Table S3. Amino acid information of GmPIN proteins are listed in Supplemental
675 Table S4.
676

677 **Rhizogenes-mediated hairy root transformation**
678 Rhizogenes-mediated hairy root transformation was carried out as described before (Kereszt et
679 al., 2007). *Agrobacterium* strain K599 carrying the constructs was directly injected into the
680 hypocotyl proximal to the cotyledon of the 5-day-old healthy soybean seedlings. The infected
681 plants were grown under high humidity condition until hairy roots emerged in high-nitrogen
682 hydroponic solution (contains 5.3 mM nitrogen including KNO_3, NH_4NO_3, $Ca(NO_3)_2 \cdot 4H_2O$,
683 (15:4:12), and 0.3 mM K_2SO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 25 µM $MgCl_2$, 2.5 µM $NaB_4O_7 \cdot 10H_2O$,
684 0.5 µM $MnSO_4 \cdot H_2O$, 1.5 µM $ZnSO_4 \cdot 7H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.15 µM
685 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 40 µM Fe-Na-EDTA, 250 µM KH_2PO_4). After the primary roots were
686 removed, the plants with the hairy roots were transferred into fresh wet vermiculite with
687 irrigation of low-nitrogen buffer, and further inoculated with rhizobium strain *BXYD3* for
688 additional 14 days growth. The positive hairy roots with dTomato fluorescence were selected
689 by hand-held lamp with 555nm excitation laser.
690

691 **Rhizobial spot inoculation**
692 5-day-old soybean seedlings were used for spot inoculation in low-nitrogen hydroponic system.
693 0.5cm root segments below the root-shoot junction were covered by humid foam with *BXYD3*
694 (OD600≅1.0) by continuous spot inoculation for 48h (equipped as shown in Fig. 5D). 3cm

695   foam (keep humid with low-nitrogen buffer) was wrapped above the inoculation site to keep

696   roots humid. Auxin concentration was measured in the above (upper, U) and below (lower, L)

697   root segments close to the inoculated site, and auxin transport was measured across the spot

698   inoculation site at 48 hpi.

699

700   **Generation of stable transgenic soybean plants**

701   For soybean stable plant transformation, the established constructs were transformed into the

702   *Agrobacterium tumefaciens* strain *EHA101* or *GV3101*. *Gmpin9d* mutant was generated in

703   Williams 82 ecotype, *Gmpin1abc* mutant and *35s:GmPIN1* lines were generated in *Huachun6*

704   ecotype. Transformants were established and screened by PCR amplification using primers for

705   basta and the corresponding genes (Supplemental Table S1).

706

707   **Histochemical GUS staining**

708   GUS staining solution contains 50 mM sodium phosphate buffer (pH 7.0), 0.1% (v/v) Triton

709   X-100, 0.1 mM $K_3Fe(CN)_6$, 0.1 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 1 mg/mL X-Gluc and 1%(v/v)

710   dimethylformamide. Samples were stained for 1-4 h at 37°C. Chlorophyll was removed by

711   sequential incubations in 50% ethanol, 100% ethanol, and 50% ethanol for several hours at

712   each step. After rehydration, samples were transferred to glass slides with 50% glycerol

713   mounted.

714

715   **Endogenous hormone measurement**

716   Phytohormone extraction and measurement were performed using HPLC as described

717   previously (Yang et al., 2019) with modifications. Briefly, root or nodules were ground to

718   powder in liquid nitrogen. Approximately 100 mg powder with the addition of a mixture of

719   internal standards were extracted with 1 mL of ethyl acetate by vortexed for 10 min and

720   sonicated for 20 min at 4 °C, respectively. After centrifugation at 12000 rpm for 3 min at 4 °C,

721   the supernatant was obtained and then evaporated until dry using a vacuum concentrator

722   (Eppendorf, Germany) at 30 °C. The dried residues were diluted in 500 μL of 70% methanol

723   and then filtered through a 0.22 mm cellulose acetate filter.

724

725   **Auxin transport measurements**

726   5-day-old seedlings were used to measure the capacity of auxin transport as described

727   previously (Lewis and Muday, 2009) with modifications. The 2cm root segments above the

728   root tip were collected (Fig. 8D). For the measurement of acropetal or basipetal auxin transport,

23

729 the bottom or top of segmented roots were inserted into the tube that contains a consistent
730 volume of small [^{3}H]-IAA agar block, for 3 h incubation in the dark. 0.5cm root segments just
731 above the inoculation site were excised and washed by 1/2 MS liquid for 15 min. Then, these
732 segments were placed into scintillation vials containing 2 mL of scintillation fluid for 16-24 h.
733 Radioactivity was measured as described previously (Lewis and Muday, 2009), and auxin
734 transport efficiency was calculated according to the method described previously (Lewis and
735 Muday, 2009).

736 For auxin transport measurement in spot inoculation system, 2.5cm root segments
737 below root-shoot junction area were collected (Fig. 5D), the top of segmented roots (U) were
738 inserted into the tube that contains a consistent amount of [^{3}H]-IAA solution, for 3 h incubation
739 in the dark. 0.5cm root segments in L position were collected for radioactivity in scintillation
740 vials containing 1 mL of scintillation fluid for 16-24 h.

741

742 **Tissue slicing and microscopy observation**
743 For resin embedding and sectioning, the different stages of nodules were collected and fixed
744 in Formalin solution, and then dehydrated in a graded alcohol series. The fixed materials were
745 subsequently embedded in Technovit 7100 (Kulzer GmbH) according to the manufacturer's
746 protocol.

747 For pPIN:GUS and PIN-GFP analysis in soybean plants, root segments were collected
748 and embedded within 7-8% agarose. After solidification, the samples were sliced using a Leica
749 RM2255 microtome in transversal or longitudinal directions of 80 μm thickness. For GFP
750 samples, the root/nodule median sections were selected under dissecting microscope with
751 obvious vasculature, and the fluorescence were further observed by confocal microscopy under
752 the same setting. For GUS samples, the slices were further stained by GUS staining solution
753 for 4-8 h, or stained with 0.001% Toluidine Blue O staining for 10-20 seconds. All samples in
754 a single experiment were stained with consistent time.

755 For fluorescence observation, images were taken by either Zeiss LSM 880 (with
756 Airyscan) or Leica SP8 confocal microscopes. The settings of excitation and detection were:
757 GFP: 488 nm, 505-550 nm; FM4-64: 535 nm, 610 nm. All images in a single experiment were
758 captured with the same setting.

759 For nodule primordium assay, 5dpi seedlings were used to quantify the density of
760 nodule primordium. The 1cm root segments were consistently collected from lateral roots in
761 root-shoot junction areas. 5 to 10 individual root segments were collected from each plant, and
762 at least 8 plants were analyzed. For nodule primordium assay in spot inoculation system, root

763   segments were collected from the foam-covered inoculated site. Root sections with 80 μm

764   thickness were produced using a vibratome (Leica). The root sections were then stained by

765   0.25% potassium permanganate for 10-30 seconds and soaked into Schiff's fuchsin-sulfite

766   solution. Nodule primordium was observed by Nikon Ti-E light microscope and the density

767   was quantified by ImageJ.

768

769   **Statistical analysis, image analysis and figure preparation**

770   Statistical data were analyzed in Graphpad Prism 7 (GraphPad Software, La Jolla California

771   USA, www.graphpad.com). Statistical images were generated by Graphpad Prism 7. Camera

772   and confocal images were prepared with ImageJ (http://imagej.nih.gov/ij/). All the experiments

773   were carried out at least in triplicate.

774

775   **Accession numbers**

776   Soybean gene accession numbers: GmPIN1a (Glyma.08G054700), GmPIN1b

777   (Glyma.07G102500), GmPIN1c (Glyma.09G176300), GmPIN1d (Glyma.03G126000),

778   GmPIN1e (Glyma.19G128800), GmPIN2a (Glyma.13G101900), GmPIN2b

779   (Glyma.17G057300), GmPIN3a (Glyma.07G217900), GmPIN3b (Glyma.20G014300),

780   GmPIN3c (Glyma.07G164600), GmPIN3d (Glyma.09G117900), GmPIN5a

781   (Glyma.09G251600), GmPIN5b (Glyma.18G241000), GmPIN6a (Glyma.13G038300),

782   GmPIN6b (Glyma.14G120900), GmPIN8a (Glyma.05G109800), GmPIN8b

783   (Glyma.17G157300), GmPIN8c (Glyma.09G240500), GmPIN8d (Glyma.18G255800),

784   GmPIN9a (Glyma.09G061800), GmPIN9b (Glyma.15G168100), GmPIN9c

785   (Glyma.09G097300), GmPIN9d (Glyma.15G208600). Arabidopsis PIN accession

786   numbers:AtPIN1(AT1G73590), AtPIN2(AT5G57090), AtPIN3(AT1G70940),

787   AtPIN4(AT2G01420), AtPIN5(AT5G16530), AtPIN6(AT1G77110), AtPIN7(AT1G23080),

788   AtPIN8(AT5G15100). KfPIN:(KJ466099).

789

790   **SUPPLEMENTAL DATA**

791   Supplemental Figure S1. NPA promotes small nodule formation.

792   Supplemental Figure S2. Structure of GmPINs.

793   Supplemental Figure S3. Expression pattern of *GmPINs*.

794   Supplemental Figure S4. Auto-fluorescence in GFP channel in WT roots and nodules.

795   Supplemental Figure S5. GmPIN1b is polarly localized toward nodule primordium apex.

796   Supplemental Figure S6. Phenotype analysis of *Gmpin1abc* triple mutant.

809

824

AUTHOR CONTRIBUTIONS

826 ZG and XC designed research; ZG, ZC, YC, MK, HX, QX, SM performed the research; ZG

827 contributed new analytic tools; YL, HZ, DH, TX and XL helped the manuscript interpretation

828 and analysed data; LH and JC generated the transgenic soybean plants; YG contributed

829 soybean CRISPR/Cas9 vector; SL, WY, JF, JP and JZ contributed the technical help and

830 helpful discussion; ZG and XC wrote the paper. ZG, ZC, YC and MK contributed equally as

831 co-first authors, and XC contributed as corresponding author.

832

833 **FIGURE LEGENDS**

834 **Figure 1. NPA disrupts auxin distribution and nodule formation.**

835 (A-B) 7-day-old WT (A) or *DR5-V2-GUS* transgenic soybean plants (B) were infected by

836 *BXYD3*, and then incubated in low-nitrogen hydroponic solution containing 10^{-7} M NPA or

837 DMSO (as mock control). Root-shoot junctions were collected in series at 4-14 dpi for resin

838 embedding and sectioning. The morphology of stage I to IV soybean nodules was examined

839 by sectioning in the longitudinal direction (A). Red boxes label the primordium area, dotted

840 lines label the vascular bundles, and black arrows highlight the nodule or nodule primordium

841 (A). *DR5-V2* activity in different nodule stages was stained and captured by sectioning in

842 longitudinal (L) or transverse (T) directions (B). St: root stele. Red dot lines label the sectioning

843 areas in transverse direction. Red arrows highlight the dividing cortical cells. Scale bars, 100

844 μm (A, B).

845 **Figure 2. Expression pattern of *GmPINs* during nodule development.**

846 (A) The phylogenetic tree of PIN proteins from Arabidopsis (At), *Glycine max* (Gm) and

847 *Klebsormidium flaccidum* (Kf) was constructed using MEGA X. These *PIN* genes were

848 grouped as canonical, non-canonical and soybean-specific PIN types.

849 (B) Transcript level of 23 *GmPIN* genes was detected by RT-qPCR in nodules at 14dpi (n≥3),

850 *Gmactin11* was the normalization gene.

851 (C) *pGmPIN1a, b, c, d, e:GUS* constructs were introduced in WT soybean plants by hairy root

852 transformation, the positive roots were infected by *BXYD3*. Root segments were collected at 4

853 to 14 dpi. Root sectioning was performed for the further histochemical staining.

854 (D) Cartoon model represents the spatial and temporal expression pattern (different colors) of

855 *GmPIN1s* during nodule development.

856 Scale bars, 100 μm (C). Error bar=S.D.

857 **Figure 3. GmPIN1 localization is conserved with AtPIN1 and direct auxin flow during**

858 **nodule primordium formation.**

859 (A) Predicted topology of GmPIN1 proteins. The following GmPIN1-GFP constructs were

860 established based on the insertion position of GFP in the hydrophilic loop.

861 (B-C) GmPIN1a-e distribution and polarity were visualized in roots of the transgenic

862 Arabidopsis plants (*pAtPIN1: GmPIN1-GFP* in *pin1-En* Arabidopsis (At.) mutant background

863 (B, C). The phenotypes of inflorescence and silique (inset boxes) were shown in C. Polar

864   localization of GmPIN1 in the basal side of PM in root stele (inset boxes) is highlighted by

865   white arrows (B).

866   (D) GmPIN1b localization was visualized in roots and different stages of nodules in the

867   transgenic soybean plant *pGmPIN1b:GmPIN1b-GFP*. The polarity of GmPIN1b (with

868   predominant polarity toward nodule apex) was highlighted by white arrows. Dot lines label the

869   area of C1-derived cell layers or vascular bundle. Pictures with bright field view were shown

870   in the inset boxes of upper panels. The images in lower panels showed the enlarged views of

871   boxed areas in the representative cartoon (1: undifferentiated vascular bundles; 2 and 3:

872   differentiated vascular bundles). Yellow and blue dotted lines represent GmPIN1b signal on

873   periclinal and anticlinal directions at stage I.

874   (E) GmPIN1b localization was visualized in nodule primordium (stage II) of NPA (10^{-7} M,

875   4days)-treated *pGmPIN1b:GmPIN1b-GFP* seedlings White arrows label GmPIN1b polarity

876   toward nodule apex.

877   (F-G) GmPIN1b signal toward the nodule apex (apical) or perpendicular to the nodule apex

878   (lateral) was individually measured in F (n=136, 189, 89, 129, 136, 189, 89, 129). The

879   comparison ratio of GmPIN1b signal in different nodule primordium stage relative to those in

880   stage I was shown as percentage in F. Polar index of GmPIN1b was quantified by calculation

881   of GmPIN1b signal ratio of apical-targeted GmPIN1b signals relative to them in the lateral

882   sides (G: n=90, 136, 189, 89, 129, 40, 102).

883   Scale bars, 5μm (B), 50μm (B, inset box), 5mm (C), 100μm (D upper panel, E), 20μm (D lower

884   panel). Error bar=S.D. In D-E: Ep, epidermis; C1, 1st cortical cell layer; C2, 2nd cortical cell

885   layer; Ed, endodermis; Pc, pericycle; St, stele; VB, vascular bundle. Root tip growth directions

886   (g) were labelled by red arrows (D, E). Nodule primordium apex (apical-targeted GmPIN1b)

887   was labelled by yellow arrows, and the lateral localization (perpendicular to the apical direction)

888   of GmPIN1b was labelled by blue arrows (D, E). P-values were determined by two-tailed

889   Student's t-test assuming equal variances (***p< 0.001; ****p<0.0001; ns, not significant).

890   **Figure 4. Nodulation phenotypes of *Gmpin1abc* triple mutant and *GmPIN1***

891   **overexpression lines.**

892   (A) *Gmpin1abc* triple mutants were generated via CRISPR-Cas9 gene editing approach. The

893   sequence of *GmPIN1a, b, c* with the gRNA targeted sites was designed in the first exon of

894   *GmPIN1* genes. gRNA targeted sequences were framed by green, and the mutation or deletion

895   sequences were labelled by red. Two independent triple mutant lines with edited genes are

896   shown.

(B-J) 7-day-old WT, *Gmpin1abc* mutants and *35s:GmPIN1a, b and c* lines were infected by *BXYD3*, and the nodulation phenotypes were analyzed at 14 dpi. Nodule number (per plant) and density (number per cm^2) were quantified in C (n=15, 9, 20, 17) and E (n=15, 9, 20, 17). Profile of nodule diameter was tracked in D (n=2138, 945), F (n=2236, 1404), H (n=848, 930, 480), I (n=1111, 471), J (n=1318, 961, 1096) (all from upper to lower groups).

Scale bars, 1cm (B, G). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (**p< 0.01; ***p< 0.001).

Figure 5. Loss-of-function and gain-of-function of *GmPIN1* disrupted auxin distribution and nodule primordium initiation.

(A-C, I) 7-day-old WT, *Gmpin1abc-L1* and *35s:GmPIN1a-L11* plants were infected by *BXYD3* for additional 5 days growth. Consistent 1cm root segments were collected from lateral roots at the root-shoot junction area. Root segments were sectioned in longitudinal direction and co-stained by Chameleon and Schiff's fuchsin-sulfite reagents (A). Nodule primordium density and developing nodule density were individually quantified as profiles in B (n=106) and C (n=106). The overall nodule primordium density was analyzed in I (n=106). The middle panels of WT in A show 3× enlarged views of boxed areas in the images of left panels.

(D,E-G,H) 0.5cm root segments below root-shoot junction of 5-day-old WT, *Gmpin1abc-L1* and *Gmpin1abc-L2* were performed by 48h continuous *BXYD3* spot inoculation (equipped as the cartoon in D). Auxin content in the upper (U) and lower (L) root segments close to the inoculated position was measured in G (uninfected roots were used as controls) (n=5, 3, 3, 4, 4, 3, 5, 5, 4, 4, 4, 4). Polar auxin transport (PAT) efficiency was measured in the acropetal direction in H (n=12, 14, 10, 13, 12, 11; red arrow in D labelled PAT direction). The comparison ratio of auxin content in L relative to those in U is shown as a percentage in G, and the comparison ratio of auxin transport capacity in infected groups to uninfected groups was shown as percentage in H. Nodule primordium morphology in WT and *Gmpin1abc* mutants was observed at 5dpi after spot inoculation (E). Cartoons simulate cell morphology of each genotype, and green color indicates the dividing cortical cells (E). The percentage in E indicates the proportion of roots with aberrant dividing cortical cells in the total counting roots.

(F) *DR5-V2-GUS* construct was introduced in WT, *Gmpin1abc-L1* and *35s:GmPIN1a-L11* plants by hairy root transformation, and different stages of nodule were collected at 8dpi for sectioning. Auxin distribution was visualized by histochemical GUS staining.

Scale bars, 200μm (A), 100μm (E, F). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (*p<0.05; **p< 0.01; ****p< 0.0001; ns, not significant).

Figure 6. Cytokinin rearranges GmPIN1b polarity.

(A-B) Nodule primordium of *BXYD3*-infected *pGmPIN1b:GmPIN1b-GFP* soybean plants were analyzed at 8dpi (with or without 1μM 6-BA treatment for 16h). GmPIN1b-GFP signal in the apical (yellow arrows) and lateral (blue arrows) directions of nodule primordium was calculated and shown as lateral/apical ratio in (B) (n=126 from 22 sections, 152 from 36 sections), and the distribution frequency of GmPIN1b signal ratio was shown in the right chart (B, grey dot line marked the cutoff value). The cells with GmPIN1b signal ratio above the cutoff value was highlighted by yellow stars (A). Root tip growth directions (g) were labelled by red arrows (A).

(C) 7-day-old WT plants were infected by *BXYD3*, and then they were irrigated with low-nitrogen hydroponic solution containing 0.1μM 6-BA or DMSO (as mock control) for additional 7 days. Cortical root cell morphology was observed by root sectioning. Numbers in the right panels showed 4× enlarged views framed in the left image . The percentage indicates the proportion of roots with a cluster of dividing cortical cells in the total counting roots.

Scale bars, 100μm (A), 500μm (C). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (****p< 0.0001).

Figure 7. *GmPIN9d* is specifically expressed in the protoxylem cells of vascular bundles and root-nodule junctions.

(A) Different tissues of 10-day-old *pGmPIN9d:GUS* transgenic soybean plants were in series collected for sectioning and histochemical staining. Xylem cells were indicated by phloroglucin co-staining. Numbers in the right and lower panels indicate the areas labelled in the left image. Pictures in the lower panels showed 3× enlarged views of the images in the upper panels.

(B) *pGmPIN9d:GUS* transgenic soybean plants were infected by *BXYD3*, roots and different stages of nodules were collected for sectioning and histochemical staining.

(C) Transcript levels of *GmPIN9a, b, c, d* genes were detected in different soybean tissues by RT-qPCR, and *Gmactin11* was used as the normalization gene. The relative transcript levels are shown by heatmap.

Scale bars, 100μm (A) and 200μm (B).

Figure 8. GmPIN9d transports auxin in an acropetal direction.

(A) *Gmpin9d* mutants were generated via CRISPR-Cas9 gene editing. gRNA targeted sites of *GmPIN9d* are framed in green, and the mutation or deletion sequences are labeled in red. Two independent mutant lines with editing manner were shown.

(B) Global morphology of 21-day-old *Gmpin9d* mutants.

965 (C) *DR5-V2-GUS* construct was introduced in WT, *Gmpin9d#2* mutant background by hairy

966 root transformation, and the positive hairy roots were infected by *BXYD3*. Roots and nodules

967 were collected at 14dpi for sectioning and histochemical staining.

968 (D-F) Auxin transport capacity of WT and *Gmpin9d* mutants was assayed in 2cm root segments

969 as described in D, and acropetal and basipetal auxin transportation were tracked by [^{3}H]-IAA

970 (n=9, 12, 6 in E; n=11, 10, 13 in F).

971 (G-H) Free IAA level was individually measured in the roots and nodules of WT and *Gmpin9d*

972 mutants (G: n=12, 15, 11; H: n=15, 15, 15).

973 (I-K) 7-day-old WT and *Gmpin9d* mutants were infected by *BXYD3*. The nodule primordium

974 density was analyzed at 6dpi (I: n=121, 137, 129), the nodule density was measured at 14dpi

975 (J: n=8, 8, 7), and the profile of nodule diameter was tracked (K: n=560, 536, 473).

976 Scale bars, 10cm (B), 100μm (C). Error bar=S.E.M in E-H, S.D. in I, J. P-values were

977 determined by two-tailed Student's t-test assuming equal variances (*p< 0.05; **p< 0.01; ns,

978 not significant).

979 **Figure 9. GmPIN9d works synergistically with GmPIN1 to coordinate nodule**

980 **enlargement.**

981 (A) GmPIN9d-GFP construct was established based on the insertion position of GFP in the

982 hydrophilic loop.

983 (B) *pGmPIN9d:GmPIN9d-GFP* construct was introduced in WT soybean plant by hairy root

984 transformation, and the positive roots were infected by *BXYD3*. Roots and nodules were

985 collected at 14dpi for sectioning, and GmPIN9d-GFP signal was visualized. Cells with polar

986 GmPIN9d are highlighted by white arrows. The numbered images show 5×enlarged views (red

987 boxes) in Supp. Fig. S11G images. Root tip growth directions are labelled by red arrows, and

988 directions of nodule apex were indicated by yellow arrows. VB, vascular bundle.

989 (C-E) WT and *Gmpin1abc* mutants were transformed by *Gmpin9d-RNAi* construct (with

990 dTomato-tag). The positive hairy roots were selected based on fluorescence and further

991 infected by *BXYD3*, and the profile of nodule diameter was tracked in D (n=613, 618, 624) at

992 14dpi. Expression level of *GmPIN9d* was detected by RT-qPCR (E, n=4), *Gmactin11* was used

993 as the normalization gene (E).

994 (F) Model for polar auxin transport in nodule primordium and developing nodule. Stage I,

995 GmPIN1 mainly directs auxin flow to the outer cortical cells, and partial auxin flows away

996 from outer cortical cells. Stage II and III: GmPIN1-dependent auxin stream flows towards the

997 nodule primordium, and some auxin is retrieved to the neighboring cells. Stage IV: auxin

998 stream is transported within vascular bundles towards nodule via GmPIN1 and GmPIN9d-

999 dependent polar auxin transport. Auxin accumulation is depicted in green, auxin gradient is
1000 indicated in light green, and the presumptive routes of auxin flow are depicted by arrows (red
1001 arrows: GmPIN1-mediation; purple arrows: GmPIN9d-mediation). Flavonoid and cytokinin
1002 act as the elicitors to mediate rapid auxin stream redirection.
1003 Scale bars, 100μm (B), 1cm (C). Error bar=S.D.
1004
1005
1006
1007

1008 References
1009

1010 **Abas, L., Kolb, M., Stadlmann, J., Janacek, D.P., Lukic, K., Schwechheimer, C., Sazanov, L.A.,**
1011   **Mach, L., Friml, J., and Hammes, U.Z.** (2021). Naphthylphthalamic acid associates with
1012   and inhibits PIN auxin transporters. Proc Natl Acad Sci U S A **118**.
1013 **Adamowski, M., and Friml, J.** (2015). PIN-dependent auxin transport: action, regulation, and
1014   evolution. Plant Cell **27,** 20-32.
1015 **Bai, M., Yuan, J., Kuang, H., Gong, P., Li, S., Zhang, Z., Liu, B., Sun, J., Yang, M., Yang, L., Wang,**
1016   **D., Song, S., and Guan, Y.** (2020). Generation of a multiplex mutagenesis population
1017   via pooled CRISPR-Cas9 in soya bean. Plant Biotechnol J **18,** 721-731.
1018 **Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., and**
1019   **Noble, W.S.** (2009). MEME SUITE: tools for motif discovery and searching. Nucleic
1020   Acids Res **37,** W202-208.
1021 **Benkova, E., and Bielach, A.** (2010). Lateral root organogenesis - from cell to organ. Curr Opin
1022   Plant Biol **13,** 677-683.
1023 **Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and**
1024   **Friml, J.** (2003). Local, efflux-dependent auxin gradients as a common module for
1025   plant organ formation. Cell **115,** 591-602.
1026 **Bozsoki, Z., Gysel, K., Hansen, S.B., Lironi, D., Kronauer, C., Feng, F., de Jong, N., Vinther, M.,**
1027   **Kamble, M., Thygesen, M.B., Engholm, E., Kofoed, C., Fort, S., Sullivan, J.T., Ronson,**
1028   **C.W., Jensen, K.J., Blaise, M., Oldroyd, G., Stougaard, J., Andersen, K.R., and**
1029   **Radutoiu, S.** (2020). Ligand-recognizing motifs in plant LysM receptors are major
1030   determinants of specificity. Science **369,** 663-670.
1031 **Brown, D.E., Rashotte, A.M., Murphy, A.S., Normanly, J., Tague, B.W., Peer, W.A., Taiz, L.,**
1032   **and Muday, G.K.** (2001). Flavonoids act as negative regulators of auxin transport in
1033   vivo in arabidopsis. Plant Physiol **126,** 524-535.
1034 **Buer, C.S., Kordbacheh, F., Truong, T.T., Hocart, C.H., and Djordjevic, M.A.** (2013). Alteration
1035   of flavonoid accumulation patterns in transparent testa mutants disturbs auxin
1036   transport, gravity responses, and imparts long-term effects on root and shoot
1037   architecture. Planta **238,** 171-189.
1038 **Cai, Z., Wang, Y., Zhu, L., Tian, Y., Chen, L., Sun, Z., Ullah, I., and Li, X.** (2017).
1039   GmTIR1/GmAFB3-based auxin perception regulated by miR393 modulates soybean
1040   nodulation. New Phytol **215,** 672-686.
1041 **Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A., and Bauer, W.D.** (1984). Anatomical
1042   analysis of the development and distribution of Rhizobium infections in soybean roots.
1043   Canadian Journal of Botany **62,** 2375-2384.
1044 **Chen, X., Irani, N.G., and Friml, J.** (2011). Clathrin-mediated endocytosis: the gateway into
1045   plant cells. Curr Opin Plant Biol **14,** 674-682.
1046 **Du, M., Spalding, E.P., and Gray, W.M.** (2020). Rapid Auxin-Mediated Cell Expansion. Annu
1047   Rev Plant Biol **71,** 379-402.
1048 **Feraru, E., and Friml, J.** (2008). PIN polar targeting. Plant Physiol **147,** 1553-1559.
1049 **Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K.** (2002). Lateral relocation
1050   of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. Nature **415,** 806-809.
1051 **Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K.**
1052   (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue.
1053   Science **282,** 2226-2230.

Gauthier-Coles, C., White, R.G., and Mathesius, U. (2018). Nodulating Legumes Are Distinguished by a Sensitivity to Cytokinin in the Root Cortex Leading to Pseudonodule Development. Front Plant Sci **9,** 1901.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell **112,** 219-230.

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc Natl Acad Sci U S A **86,** 1244-1248.

Ip, H., D'Aoust, F., Begum, A.A., Zhang, H., Smith, D.L., Driscoll, B.T., and Charles, T.C. (2001). Bradyrhizobium japonicum mutants with enhanced sensitivity to genistein resulting in altered nod gene regulation. Mol Plant Microbe Interact **14,** 1404-1410.

Kereszt, A., Li, D., Indrasumunar, A., Nguyen, C.D., Nontachaiyapoom, S., Kinkema, M., and Gresshoff, P.M. (2007). Agrobacterium rhizogenes-mediated transformation of soybean to study root biology. Nat Protoc **2,** 948-952.

Kohlen, W., Ng, J.L.P., Deinum, E.E., and Mathesius, U. (2018). Auxin transport, metabolism, and signalling during nodule initiation: indeterminate and determinate nodules. J Exp Bot **69,** 229-244.

Kuhn, B.M., Nodzynski, T., Errafi, S., Bucher, R., Gupta, S., Aryal, B., Dobrev, P., Bigler, L., Geisler, M., Zazimalova, E., Friml, J., and Ringli, C. (2017). Flavonol-induced changes in PIN2 polarity and auxin transport in the Arabidopsis thaliana rol1-2 mutant require phosphatase activity. Sci Rep **7,** 41906.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol **35,** 1547-1549.

Kurepa, J., Shull, T.E., and Smalle, J.A. (2019). Antagonistic activity of auxin and cytokinin in shoot and root organs. Plant Direct **3,** e00121.

Laffont, C., Ivanovici, A., Gautrat, P., Brault, M., Djordjevic, M.A., and Frugier, F. (2020). The NIN transcription factor coordinates CEP and CLE signaling peptides that regulate nodulation antagonistically. Nat Commun **11,** 3167.

Lang, K., Lindemann, A., Hauser, F., and Gottfert, M. (2008). The genistein stimulon of Bradyrhizobium japonicum. Mol Genet Genomics **279,** 203-211.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. Bioinformatics **23,** 2947-2948.

Lewis, D.R., and Muday, G.K. (2009). Measurement of auxin transport in Arabidopsis thaliana. Nat Protoc **4,** 437-451.

Liao, C.Y., Smet, W., Brunoud, G., Yoshida, S., Vernoux, T., and Weijers, D. (2015). Reporters for sensitive and quantitative measurement of auxin response. Nat Methods **12,** 207-210, 202 p following 210.

Liu, C.W., and Murray, J.D. (2016). The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. Plants (Basel) **5,** 33.

Livingston, D., Tuong, T., Nogueira, M., and Sinclair, T. (2019). Three-dimensional reconstruction of soybean nodules provides an update on vascular structure. Am J Bot **106,** 507-513.

Marhavy, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., and Benkova, E. (2014). Cytokinin controls polarity of PIN1-dependent auxin transport during lateral root organogenesis. Curr Biol **24,** 1031-1037.

Mashiguchi, K., Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., McSteen, P., Zhao, Y., Hayashi, K., Kamiya, Y., and Kasahara, H. (2011). The main auxin biosynthesis pathway in Arabidopsis. Proc Natl Acad Sci U S A **108,** 18512-18517.

Mathesius, U., Schlaman, H.R., Spaink, H.P., Of Sautter, C., Rolfe, B.G., and Djordjevic, M.A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. The Plant journal : for cell and molecular biology **14,** 23-34.

Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P., Chand, S., Benkova, E., Zazimalova, E., and Friml, J. (2008). Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development **135,** 3345-3354.

Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. EMBO J **17,** 6903-6911.

Murray, J.D., Karas, B.J., Sato, S., Tabata, S., Amyot, L., and Szczyglowski, K. (2007). A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. Science **315,** 101-104.

Ng, J.L., Hassan, S., Truong, T.T., Hocart, C.H., Laffont, C., Frugier, F., and Mathesius, U. (2015). Flavonoids and Auxin Transport Inhibitors Rescue Symbiotic Nodulation in the Medicago truncatula Cytokinin Perception Mutant cre1. Plant Cell **27,** 2210-2226.

Ng, J.L.P., and Mathesius, U. (2018). Acropetal Auxin Transport Inhibition Is Involved in Indeterminate But Not Determinate Nodule Formation. Front Plant Sci **9,** 169.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. Plant Cell **3,** 677-684.

Pacios-Bras, C., Schlaman, H.R., Boot, K., Admiraal, P., Langerak, J.M., Stougaard, J., and Spaink, H.P. (2003). Auxin distribution in Lotus japonicus during root nodule development. Plant Mol Biol **52,** 1169-1180.

Peer, W.A., Bandyopadhyay, A., Blakeslee, J.J., Makam, S.N., Chen, R.J., Masson, P.H., and Murphy, A.S. (2004). Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in Arabidopsis thaliana. Plant Cell **16,** 1898-1911.

Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazimalova, E., and Friml, J. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. Science **312,** 914-918.

Pierre-Jerome, E., Drapek, C., and Benfey, P.N. (2018). Regulation of Division and Differentiation of Plant Stem Cells. Annu Rev Cell Dev Biol **34,** 289-310.

Popp, C., and Ott, T. (2011). Regulation of signal transduction and bacterial infection during root nodule symbiosis. Curr Opin Plant Biol **14,** 458-467.

Qi, J., Wang, Y., Yu, T., Cunha, A., Wu, B., Vernoux, T., Meyerowitz, E., and Jiao, Y. (2014). Auxin depletion from leaf primordia contributes to organ patterning. Proc Natl Acad Sci U S A **111,** 18769-18774.

Reid, D., Nadzieja, M., Novak, O., Heckmann, A.B., Sandal, N., and Stougaard, J. (2017). Cytokinin Biosynthesis Promotes Cortical Cell Responses during Nodule Development. Plant Physiol **175,** 361-375.

Rightmyer, A.P., and Long, S.R. (2011). Pseudonodule formation by wild-type and symbiotic mutant Medicago truncatula in response to auxin transport inhibitors. Mol Plant Microbe Interact **24,** 1372-1384.

Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L., Klein, M., Bailly, A., Lee, Y., Friml, J., Geisler, M., and Martinoia, E. (2008). Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses. J Biol Chem **283,** 31218-31226.

Schaller, G.E., Bishopp, A., and Kieber, J.J. (2015). The yin-yang of hormones: cytokinin and auxin interactions in plant development. Plant Cell **27,** 44-63.

Schiessl, K., Lilley, J.L.S., Lee, T., Tamvakis, I., Kohlen, W., Bailey, P.C., Thomas, A., Luptak, J., Ramakrishnan, K., Carpenter, M.D., Mysore, K.S., Wen, J., Ahnert, S., Grieneisen, V.A., and Oldroyd, G.E.D. (2019). NODULE INCEPTION Recruits the Lateral Root Developmental Program for Symbiotic Nodule Organogenesis in Medicago truncatula. Curr Biol **29,** 3657-3668 e3655.

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W., Guo, H., Ljung, K., and Alonso, J.M. (2011). The Arabidopsis YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. Plant Cell **23,** 3961-3973.

Subramanian, S., Stacey, G., and Yu, O. (2006). Endogenous isoflavones are essential for the establishment of symbiosis between soybean and Bradyrhizobium japonicum. The Plant journal : for cell and molecular biology **48,** 261-273.

Subramanian, S., Stacey, G., and Yu, O. (2007). Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci **12,** 282-285.

Suzaki, T., and Kawaguchi, M. (2014). Root nodulation: a developmental program involving cell fate conversion triggered by symbiotic bacterial infection. Curr Opin Plant Biol **21,** 16-22.

Suzaki, T., Yano, K., Ito, M., Umehara, Y., Suganuma, N., and Kawaguchi, M. (2012). Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response. Development **139,** 3997-4006.

Teale, W.D., Pasternak, T., Dal Bosco, C., Dovzhenko, A., Kratzat, K., Bildl, W., Schworer, M., Falk, T., Ruperti, B., Schaefer, J.V., Shahriari, M., Pilgermayer, L., Li, X., Lubben, F., Pluckthun, A., Schulte, U., and Palme, K. (2021). Flavonol-mediated stabilization of PIN efflux complexes regulates polar auxin transport. EMBO J **40,** e104416.

Turner, M., Nizampatnam, N.R., Baron, M., Coppin, S., Damodaran, S., Adhikari, S., Arunachalam, S.P., Yu, O., and Subramanian, S. (2013). Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiol **162,** 2042-2055.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell **9,** 1963-1971.

van Noorden, G.E., Kerim, T., Goffard, N., Wiblin, R., Pellerone, F.I., Rolfe, B.G., and Mathesius, U. (2007). Overlap of proteome changes in Medicago truncatula in response to auxin and Sinorhizobium meliloti. Plant Physiol **144,** 1115-1131.

1193 **Viaene, T., Delwiche, C.F., Rensing, S.A., and Friml, J.** (2013). Origin and evolution of PIN
1194   auxin transporters in the green lineage. Trends Plant Sci **18,** 5-10.

1195 **Wang, Y., Chai, C., Valliyodan, B., Maupin, C., Annen, B., and Nguyen, H.T.** (2015a). Genome-
1196   wide analysis and expression profiling of the PIN auxin transporter gene family in
1197   soybean (Glycine max). BMC Genomics **16,** 951.

1198 **Wang, Y., Yang, W., Zuo, Y., Zhu, L., Hastwell, A.H., Chen, L., Tian, Y., Su, C., Ferguson, B.J.,**
1199   **and Li, X.** (2019). GmYUC2a mediates auxin biosynthesis during root development and
1200   nodulation in soybean. J Exp Bot **70,** 3165-3176.

1201 **Wang, Y., Li, K., Chen, L., Zou, Y., Liu, H., Tian, Y., Li, D., Wang, R., Zhao, F., Ferguson, B.J.,**
1202   **Gresshoff, P.M., and Li, X.** (2015b). MicroRNA167-Directed Regulation of the Auxin
1203   Response Factors GmARF8a and GmARF8b Is Required for Soybean Nodulation and
1204   Lateral Root Development. Plant Physiol **168,** 984-999.

1205 **Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P., and**
1206   **Offringa, R.** (2005). Maintenance of embryonic auxin distribution for apical-basal
1207   patterning by PIN-FORMED-dependent auxin transport in Arabidopsis. Plant Cell **17,**
1208   2517-2526.

1209 **Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D.,**
1210   **Benkova, E., Scheres, B., and Friml, J.** (2006). Polar PIN localization directs auxin flow
1211   in plants. Science **312,** 883.

1212 **Wu, C., Dickstein, R., Cary, A.J., and Norris, J.H.** (1996). The Auxin Transport Inhibitor N-(1-
1213   Naphthyl)phthalamic Acid Elicits Pseudonodules on Nonnodulating Mutants of White
1214   Sweetclover. Plant Physiol **110,** 501-510.

1215 **Wu, M.F., Yamaguchi, N., Xiao, J., Bargmann, B., Estelle, M., Sang, Y., and Wagner, D.** (2015).
1216   Auxin-regulated chromatin switch directs acquisition of flower primordium founder
1217   fate. Elife **4,** e09269.

1218 **Xiao, T.T., Schilderink, S., Moling, S., Deinum, E.E., Kondorosi, E., Franssen, H., Kulikova, O.,**
1219   **Niebel, A., and Bisseling, T.** (2014). Fate map of Medicago truncatula root nodules.
1220   Development **141,** 3517-3528.

1221 **Xiong, Y., and Jiao, Y.** (2019). The Diverse Roles of Auxin in Regulating Leaf Development.
1222   Plants (Basel) **8,** 243.

1223 **Xu, J., and Scheres, B.** (2005). Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1
1224   function in epidermal cell polarity. Plant Cell **17,** 525-536.

1225 **Yamaguchi, N., Wu, M.F., Winter, C.M., Berns, M.C., Nole-Wilson, S., Yamaguchi, A.,**
1226   **Coupland, G., Krizek, B.A., and Wagner, D.** (2013). A molecular framework for auxin-
1227   mediated initiation of flower primordia. Dev Cell **24,** 271-282.

1228 **Yang, H., Wang, Y., Li, L., Li, F., He, Y., Wu, J., and Wei, C.** (2019). Transcriptomic and
1229   Phytochemical Analyses Reveal Root-Mediated Resource-Based Defense Response to
1230   Leaf Herbivory by Ectropis oblique in Tea Plant (Camellia sinensis). J Agric Food Chem
1231   **67,** 5465-5476.

1232 **Zhang, J., Subramanian, S., Stacey, G., and Yu, O.** (2009). Flavones and flavonols play distinct
1233   critical roles during nodulation of Medicago truncatula by Sinorhizobium meliloti. The
1234   Plant journal : for cell and molecular biology **57,** 171-183.

1235 **Zhang, J., Nodzynski, T., Pencik, A., Rolcik, J., and Friml, J.** (2010). PIN phosphorylation is
1236   sufficient to mediate PIN polarity and direct auxin transport. Proc Natl Acad Sci U S A
1237   **107,** 918-922.

1238 **Zhang, X., Huang, X., Li, Y., Tao, F., Zhao, Q., and Li, W.** (2021). Polar auxin transport May Be
1239   responsive to specific features of flavonoid structure. Phytochemistry **185,** 112702.

37

1240   **Zhao, Y.** (2018). Essential Roles of Local Auxin Biosynthesis in Plant Development and in
1241   Adaptation to Environmental Changes. Annu Rev Plant Biol **69,** 417-435.
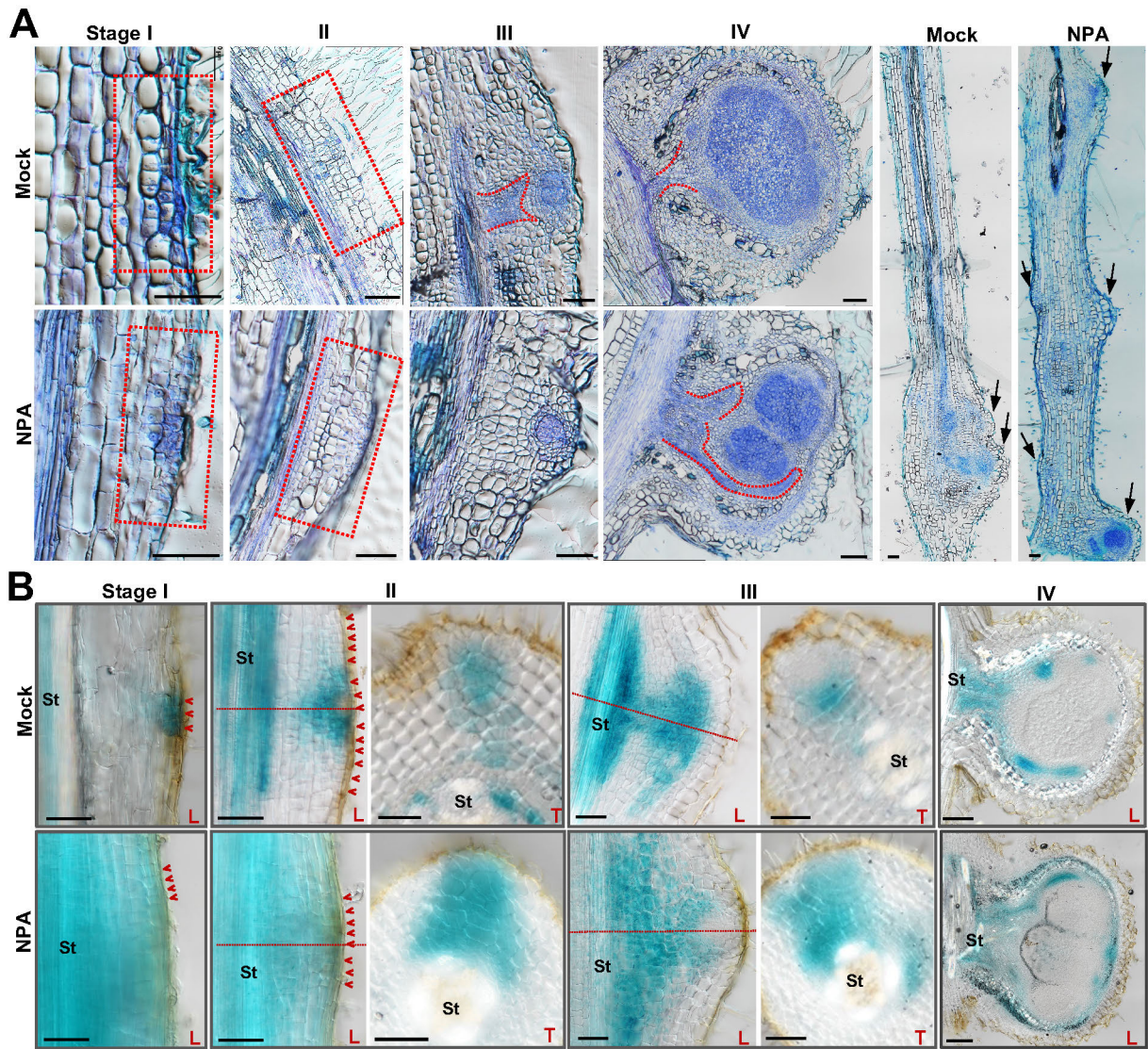1242

Figure 1. NPA disrupts auxin distribution and nodule formation.

(A-B) 7-day-old WT (A) or *DR5-V2-GUS* transgenic soybean plants (B) were infected by *BXYD3*, and then incubated in low-nitrogen hydroponic solution containing 10^{-7} M NPA or DMSO (as mock control). Root-shoot junction area were collected in series at 4dpi till 14dpi for resin embedding and sectioning. The morphology of soybean nodules in stage I to IV was sectioned in longitudinal direction (A). Red boxes label the primordium area, dotted lines label the vascular bundles, and black arrows highlight nodule or nodule primordium (A). *DR5-V2* activity in different nodule stages was stained and captured by sectioning in longitudinal (L) or transversal (T) direction (B). St: root stele. Red dot lines label the sectioning areas in transversal direction. Red arrows highlight the dividing cortical cells. Scale bars, 100 μm (A, B).
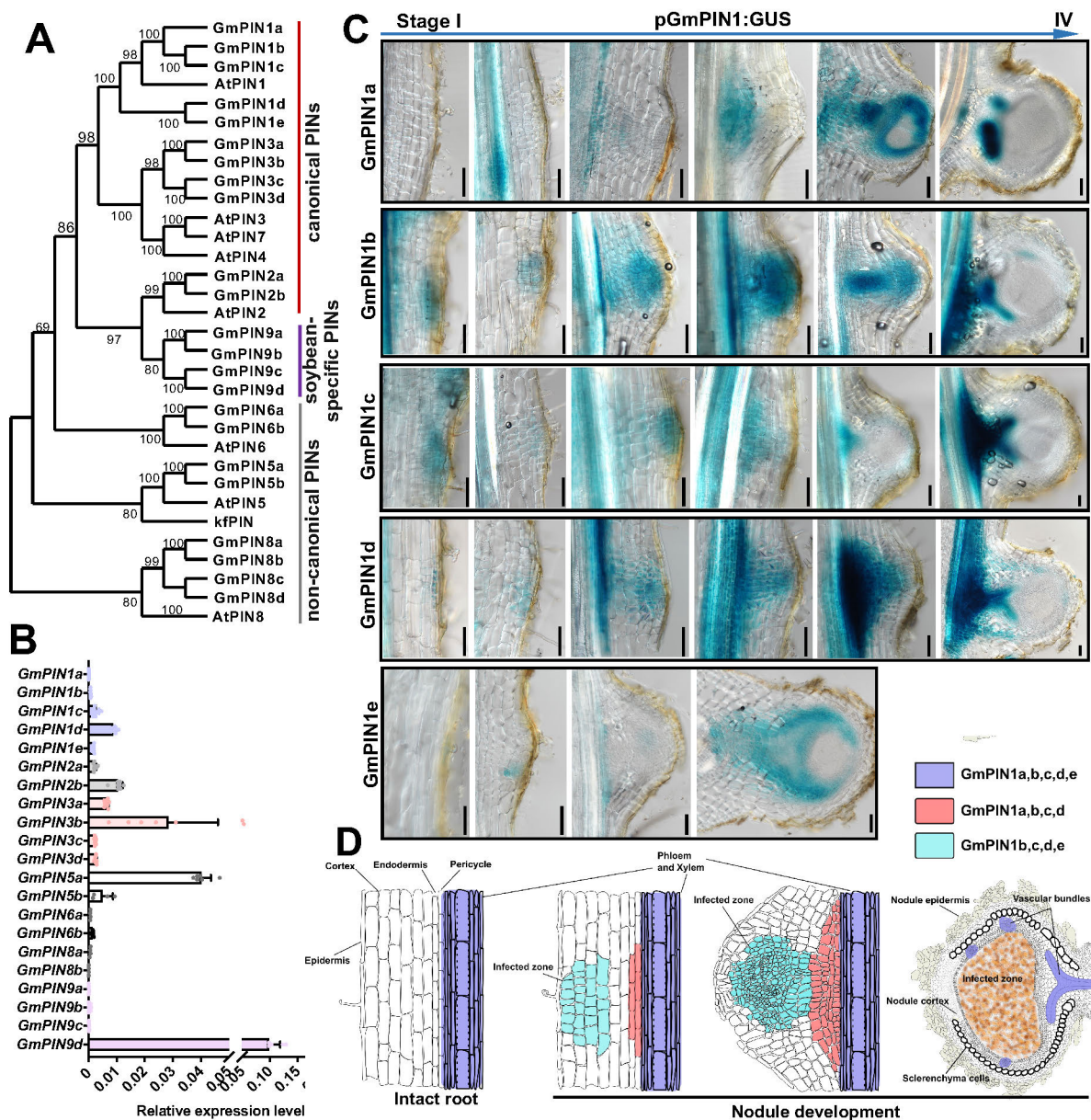
Figure 2. Expression pattern of *GmPINs* during nodule development.

(A) The phylogenetic tree of PIN proteins from *Arabidopsis* (At), *Glycine max* (Gm) and *Klebsormidium flaccidum* (Kf) was constructed using MEGA X. These *PIN* genes were grouped as canonical, non-canonical and soybean-specific PIN types.

(B) Transcript level of 23 *GmPIN* genes was detected by RT-qPCR in nodules at 14dpi (n≥3), *Gmactin11* was the normalization gene.

(C) *pGmPIN1a, b, c, d, e:GUS* constructs were introduced in WT soybean plants by hairy root transformation, the positive roots were infected by *BXYD3*. Root segments were collected at 4dpi till 14dpi. Root sectioning was performed for the further histochemical staining.

(D) Cartoon model represents the spatial and temporal expression pattern (different colors) of *GmPIN1s* during nodule development.

Scale bars, 100μm (C). Error bar=S.D.
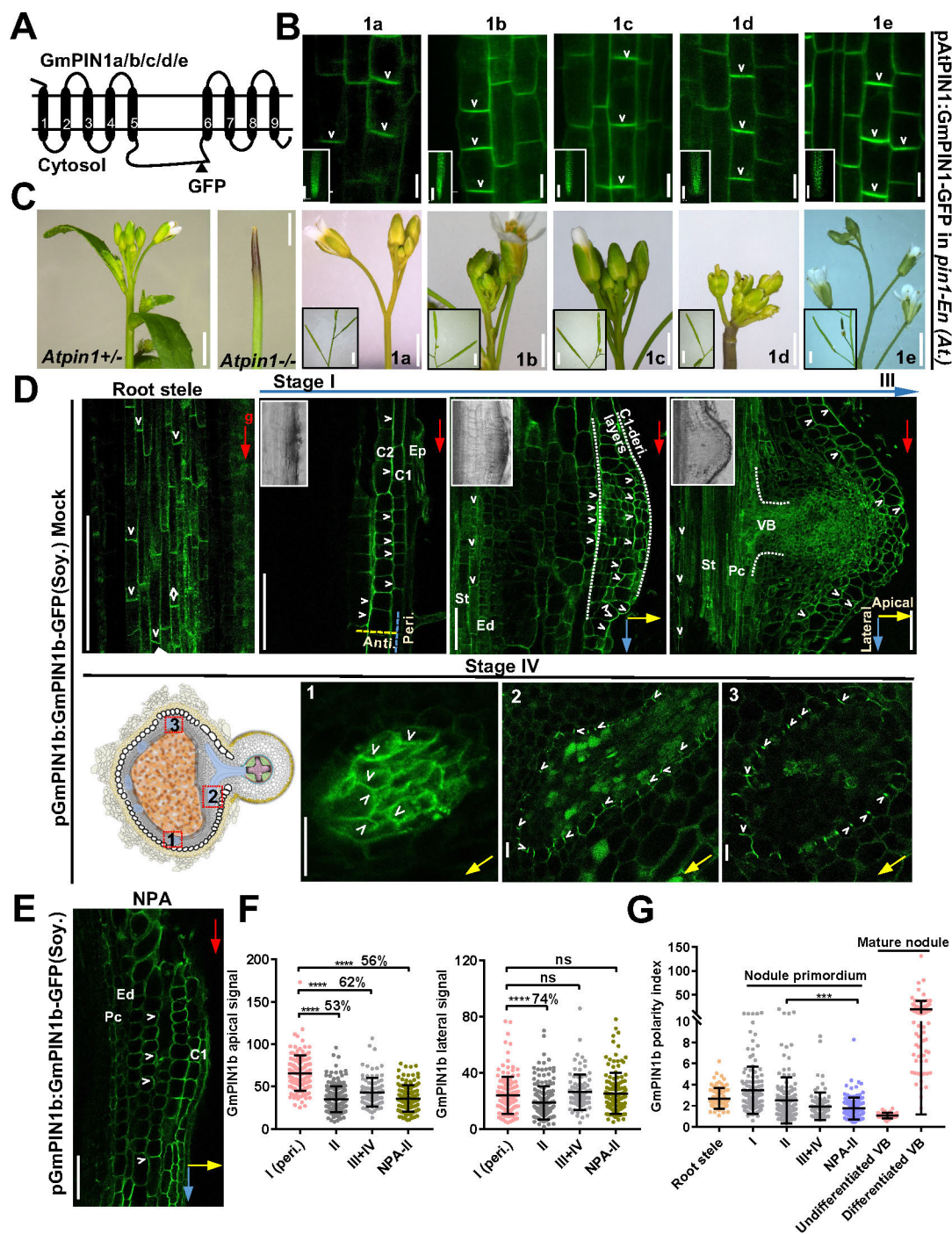
Figure 3. GmPIN1 are localization conserved with AtPIN1 and direct auxin flow during nodule primordium formation.

(A) Predicted topology of GmPIN1 proteins. The following GmPIN1-GFP constructs were established based on the insertion position of GFP in the hydrophilic loop.

(B-C) GmPIN1a-e distribution and polarity were visualized in roots of the transgenic *Arabidopsis* plants (*pAtPIN1: GmPIN1-GFP* in *pin1-En Arabidopsis (At.)* mutant background (B, C). The phenotypes of inflorescence and silique (inset boxes) were shown in C. Polar localization of GmPIN1 in the basal side of PM in root stele (inset boxes) was highlighted by white arrows (B).

(D) GmPIN1b localization was visualized in roots and different stages of nodules in the transgenic soybean plant *pGmPIN1b:GmPIN1b-GFP*. The polarity of GmPIN1b (with predominant polarity toward nodule apex) was highlighted by white arrows. Dot lines label the area of C1-derived cell layers or vascular bundle. Pictures with bright field view were shown in the inset boxes of upper panels. The images in lower panels showed the enlarged views of boxed areas in the representative cartoon (1: undifferentiated vascular bundles; 2 and 3: differentiated vascular bundles). Yellow and blue dot lines represent GmPIN1b signal on periclinal and anticlinal directions at stage I.

(E) GmPIN1b localization was visualized in nodule primordium (stage II) of NPA (10^{-7} M, 4days)-treated *pGmPIN1b:GmPIN1b-GFP* seedlings White arrows label GmPIN1b polarity toward nodule apex.

(F-G) GmPIN1b signal toward nodule apex (apical) or perpendicular to nodule apex (lateral) was individually measured in F (n=136, 189, 89, 129, 136, 189, 89, 129). The comparison ratio of GmPIN1b signal in different nodule primordium stage relative to those in stage I was shown as percentage in F. Polar index of GmPIN1b was quantified by calculation of GmPIN1b signal ratio of apical-targeted GmPIN1b signals relative to them in the lateral sides (G: n=90, 136, 189, 89, 129, 40, 102).

Scale bars, 5μm (B), 50μm (B, inset box), 5mm (C), 100μm (D upper panel, E), 20μm (D lower panel). Error bar=S.D. In D-E: Ep, epidermis; C1, 1st cortical cell layer; C2, 2nd cortical cell layer; Ed, endodermis; Pc, pericycle; St, stele; VB, vascular bundle. Root tip growth directions (g) were labelled by red arrows (D, E). Nodule primordium apex (apical-targeted GmPIN1b) was labelled by yellow arrows, and the lateral localization (perpendicular to the apical direction) of GmPIN1b was labelled by blue arrows (D, E). P-values were determined by two-tailed Student's t-test assuming equal variances (***p< 0.001; ****p<0.0001; ns, not significant).
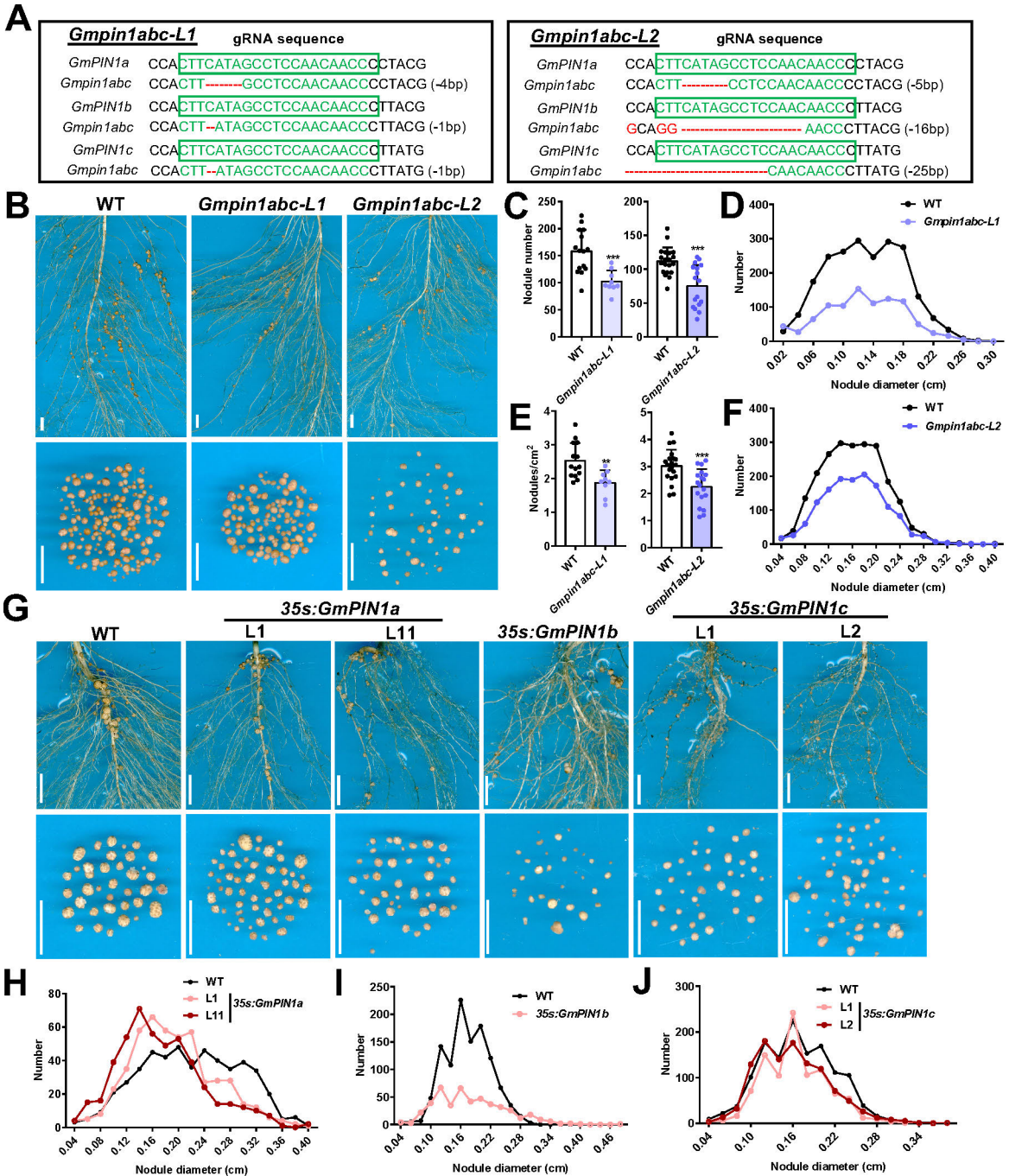
Figure 4. Nodulation phenotypes of *Gmpin1abc* triple mutant and overexpressed *GmPIN1* lines.

(A) *Gmpin1abc* triple mutants were generated via CRISPR-Cas9 gene editing approach. The sequence of *GmPIN1a, b, c* with the gRNA targeted sites was designed in the first exon of *GmPIN1* genes. gRNA targeted sequences were framed by green, and the mutation or deletion sequences were labelled by red. Two independent triple mutant lines with editing manner were shown.

(B-J) 7-day-old WT, *Gmpin1abc* mutants and *35s:GmPIN1a, b and c* lines were infected by *BXYD3*, and the nodulation phenotypes were analyzed at 14 dpi. Nodule number (per plant) and density (number per cm²) were quantified in C (n=15, 9, 20, 17) and E (n=15, 9, 20, 17). Profile of nodule diameter was tracked in D (n=2138, 945), F (n=2236, 1404), H (n=848, 930, 480), I (n=1111, 471), J (n=1318, 961, 1096) (all from upper to lower groups).

Scale bars, 1cm (B, G). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (**p< 0.01; ***p< 0.001).
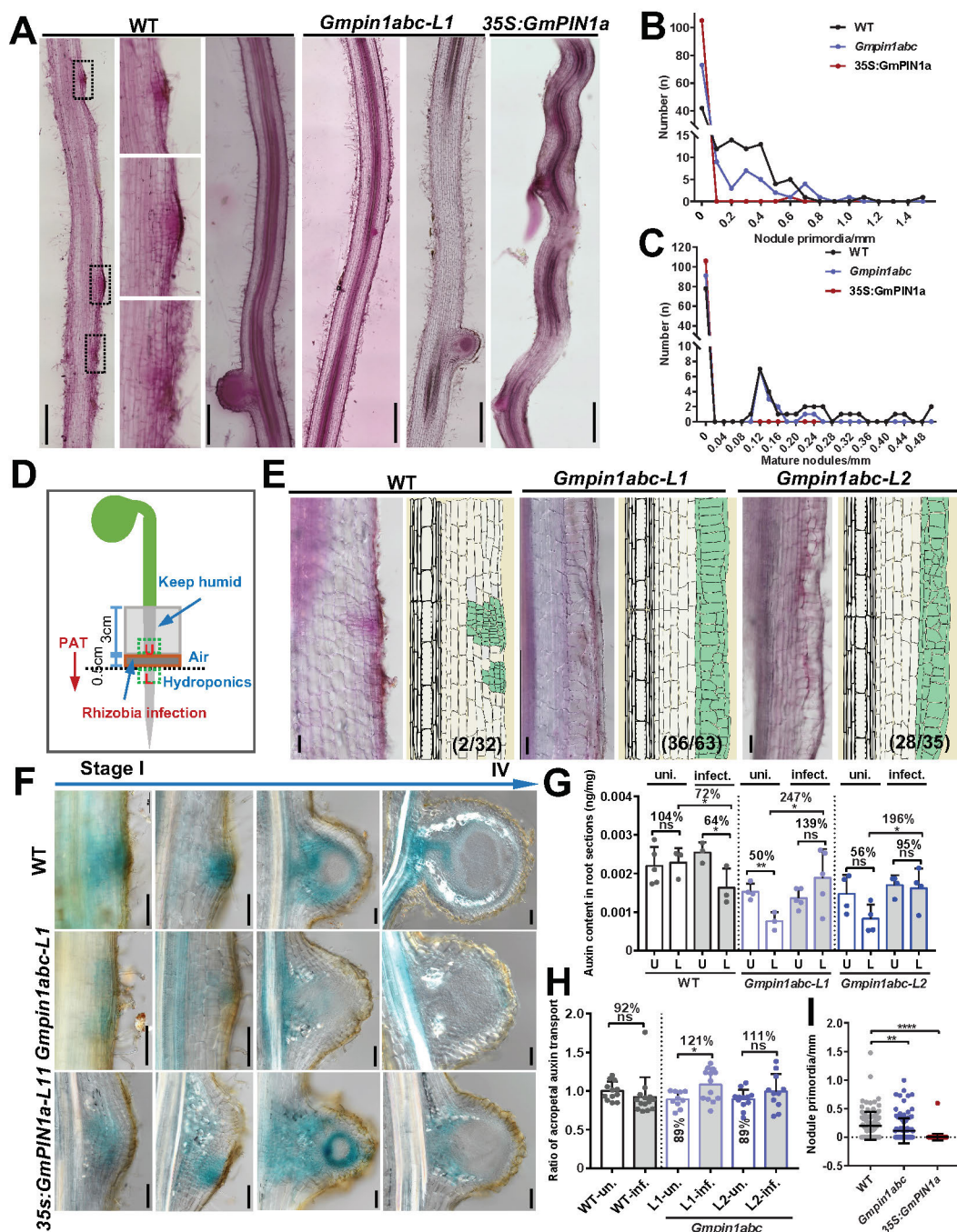
Figure 5. Loss-of-function and gain-of-function of *GmPIN1* both disrupted auxin distribution and nodule primordium initiation.

(A-C, I) 7-day-old WT, *Gmpin1abc-L1* and *35s:GmPIN1a-L11* plants were infected by *BXYD3* for additional 5 days growth. Consistent 1cm root segments were collected from lateral roots at root-shoot junction area. Root segments were sectioned in longitudinal direction and co-stained by chameleon and schiff's fuchsin-sulfite reagents (A). Nodule primordium density and developing nodule density were individually quantified as profile in B (n=106) and C (n=106). The overall nodule primordium density was analyzed in I (n=106). The middle panels of WT in A showed 3× enlarged views of boxed areas in the images of left panels.

(D,E-G,H) 0.5cm root segments below root-shoot junction of 5-day-old WT, *Gmpin1abc-L1* and *Gmpin1abc-L2* were performed by 48h continuous *BXYD3* spot inoculation (equipped as the cartoon in D). Auxin content in the upper (U) and lower (L) root segments close to the inoculated position was measured in G (uninfected roots were used as controls) (n=5, 3, 3, 4, 4, 3, 5, 5, 4, 4, 4, 4). Polar auxin transport (PAT) efficiency was measured in acropetal direction in H (n=12, 14, 10, 13, 12, 11; red arrow in D labelled PAT direction). The comparison ratio of auxin content in L relative to those in U was shown as percentage in G, and the comparison ratio of auxin transport capacity in infected groups to uninfected groups was shown as percentage in H. Nodule primordium morphology in WT and *Gmpin1abc* mutants was observed at 5dpi after spot inoculation (E). Cartoons simulate cell morphology of each genotype, and green color indicates the dividing cortical cells (E). The percentage in E indicated the proportion of roots with aberrant dividing cortical cells in the total counting roots.

(F) *DR5-V2-GUS* construct was introduced in WT, *Gmpin1abc-L1* and *35s:GmPIN1a-L11* plants by hairy root transformation, and different stages of nodule were collected at 8dpi for sectioning. Auxin distribution was visualized by histochemical GUS staining.

Scale bars, 200µm (A), 100µm (E, F). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (*p<0.05; **p< 0.01; ****p< 0.0001; ns, not significant).
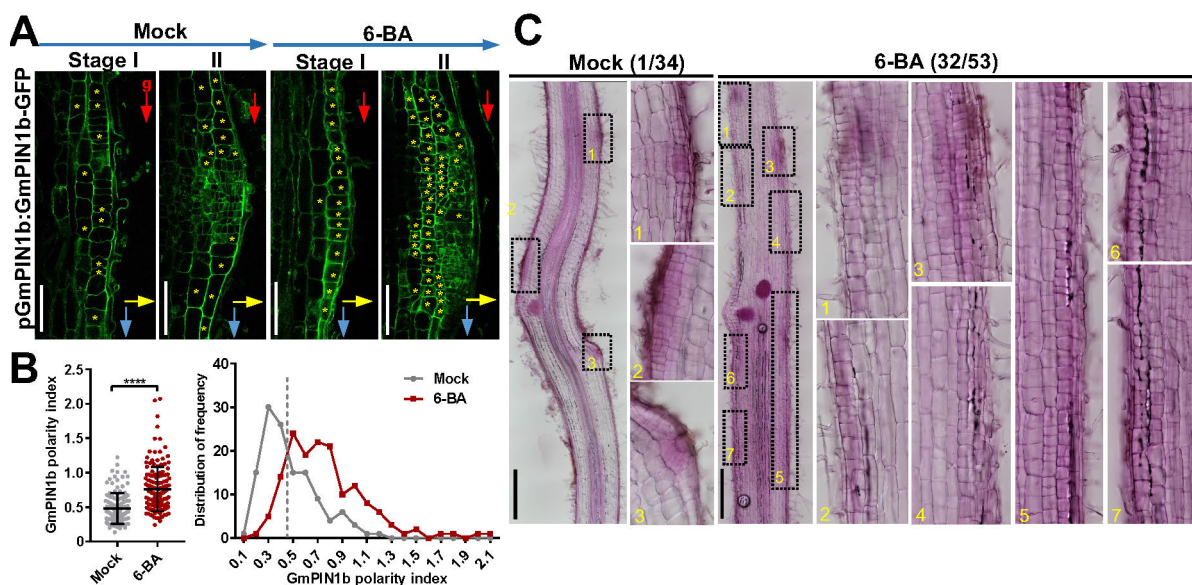
Figure 6. Cytokinin rearranges GmPIN1b polarity.

(A-B) Nodule primordium of *BXYD3*-infected *pGmPIN1b:GmPIN1b-GFP* soybean plants were analyzed at 8dpi (with or without 1μM 6-BA treatment for 16h). GmPIN1b-GFP signal in the apical (yellow arrows) and lateral (blue arrows) directions of nodule primordium was calculated and shown as lateral/apical ratio in (B) (n=126 from 22 sections, 152 from 36 sections), and the distribution frequency of GmPIN1b signal ratio was shown in the right chart (B, grey dot line marked the cutoff value). The cells with GmPIN1b signal ratio above the cutoff value was highlighted by yellow stars (A). Root tip growth directions (g) were labelled by red arrows (A).

(C) 7-day-old WT plants were infected by *BXYD3*, and then they were irrigated with low-nitrogen hydroponic solution containing 0.1μM 6-BA or DMSO (as mock control) for additional 7 days. Cortical root cell morphology was observed by root sectioning. Numbers in the right panels showed 4× enlarged views framed in the left image. The percentage indicated the proportion of roots with a cluster of dividing cortical cells in the total counting roots.

Scale bars, 100μm (A), 500μm (C). Error bar=S.D. P-values were determined by two-tailed Student's t-test assuming equal variances (****p< 0.0001).
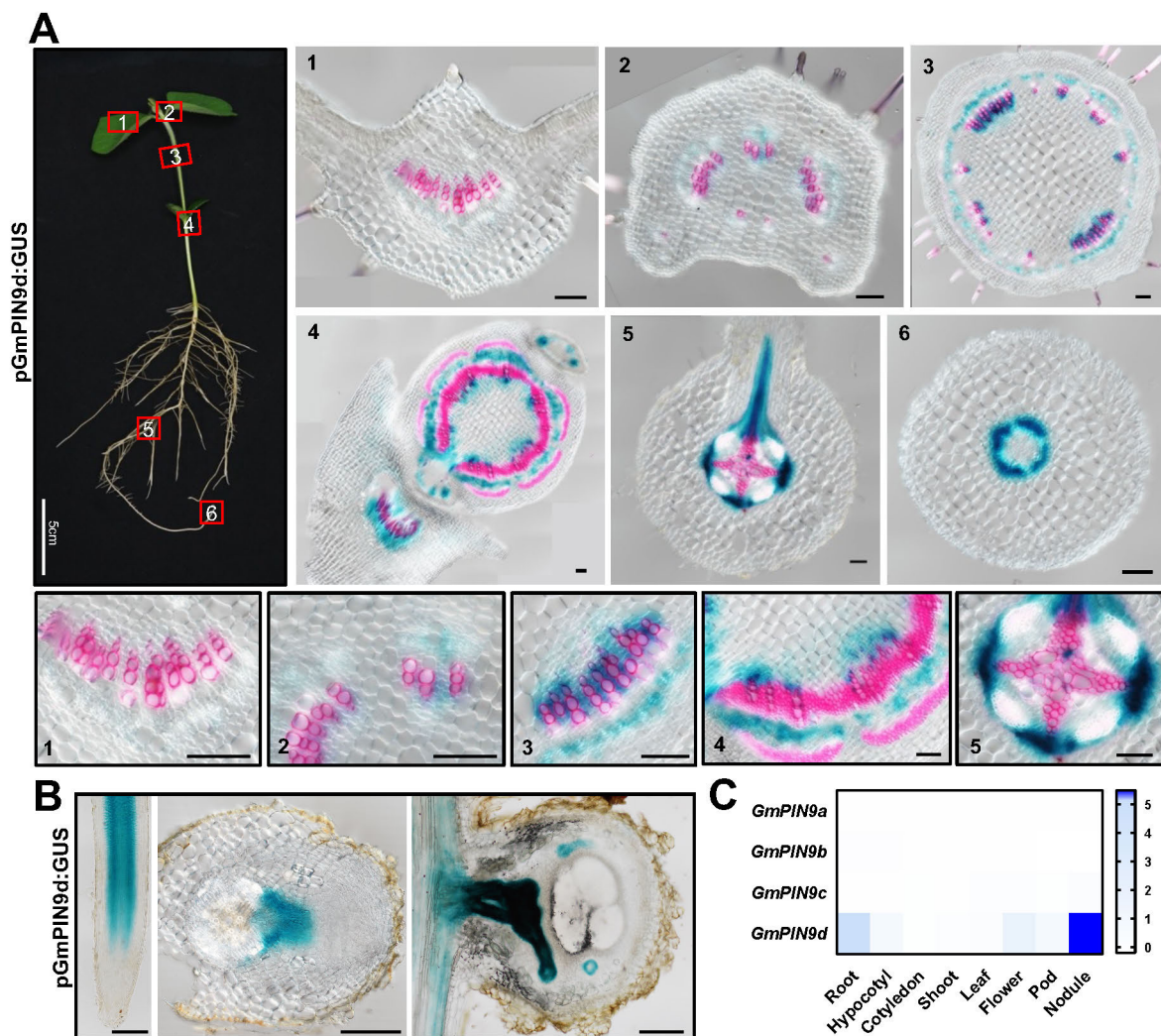
Figure 7. *GmPIN9d* **is specifically expressed in the protoxylem cells of vascular bundles and root-nodule junctions.**

(A) Different tissues of 10-day-old *pGmPIN9d:GUS* transgenic soybean plants were in series collected for sectioning and histochemical staining. Xylem cells were indicated by phloroglucin co-staining. Numbers in the right and lower panels indicated the areas labelled in the left image. Pictures in the lower panels showed 3× enlarged views of the images in the upper panels.

(B) *pGmPIN9d:GUS* transgenic soybean plants were infected by *BXYD3*, roots and different stages of nodules were collected for sectioning and histochemical staining.

(C) Transcript level of *GmPIN9a, b, c, d* genes was detected in different soybean tissues by RT-qPCR, and *Gmactin11* was used as the normalization gene. The relative transcript was shown by heatmap.
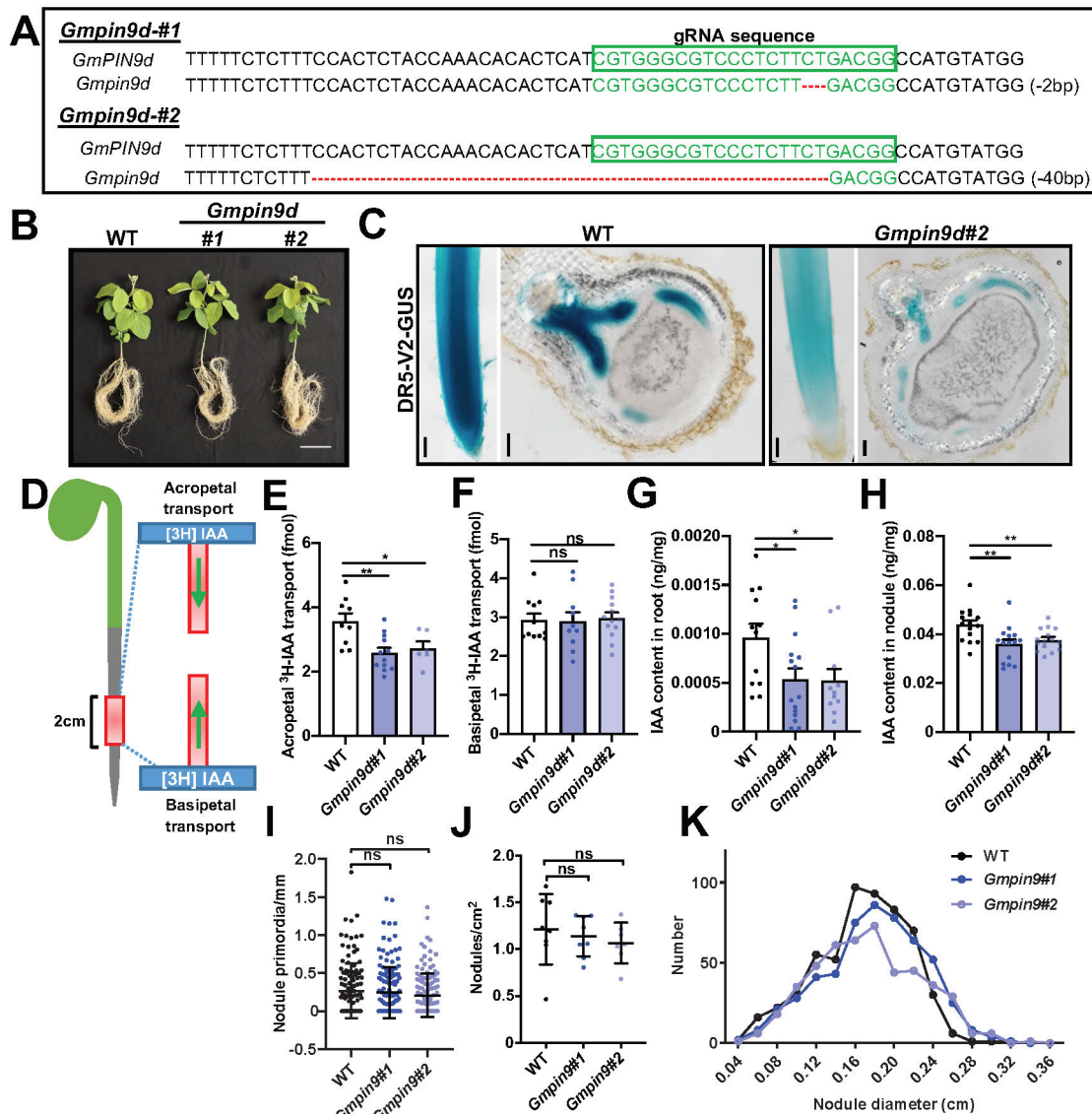
Scale bars, 100μm (A) and 200μm (B).

Figure 8. GmPIN9d transports auxin in an acropetal direction.

(A) *Gmpin9d* mutants were generated via CRISPR-Cas9 gene editing approach. gRNA targeted sites of *GmPIN9d* were framed by green, and the mutation or deletion sequences were labelled by red. Two independent mutant lines with editing manner were shown.

(B) Global morphology of 21-day-old *Gmpin9d* mutants.

(C) *DR5-V2-GUS* construct was introduced in WT, *Gmpin9d#2* mutant background by hairy root transformation, and the positive hairy roots were infected by *BXYD3.* Roots and nodules were collected at 14dpi for sectioning and histochemical staining.

(D-F) Auxin transport capacity of WT and *Gmpin9d* mutants was assayed in 2cm root segments as described in D, and acropetal and basipetal auxin transportation were tracked by [^{3}H]-IAA (n=9, 12, 6 in E; n=11, 10, 13 in F).

(G-H) Free IAA level was individually measured in the roots and nodules of WT and *Gmpin9d* mutants (G: n=12, 15, 11; H: n=15, 15, 15).

(I-K) 7-day-old WT and *Gmpin9d* mutants were infected by *BXYD3*. The nodule primordium density was analyzed at 6dpi (I: n=121, 137, 129), the nodule density was measured at 14dpi (J: n=8, 8, 7), and the profile of nodule diameter was tracked (K: n=560, 536, 473).

Scale bars, 10cm (B), 100μm (C). Error bar=S.E.M in E-H, S.D. in I, J. P-values were determined by two-tailed Student's t-test assuming equal variances (*p< 0.05; **p< 0.01; ns, not significant).
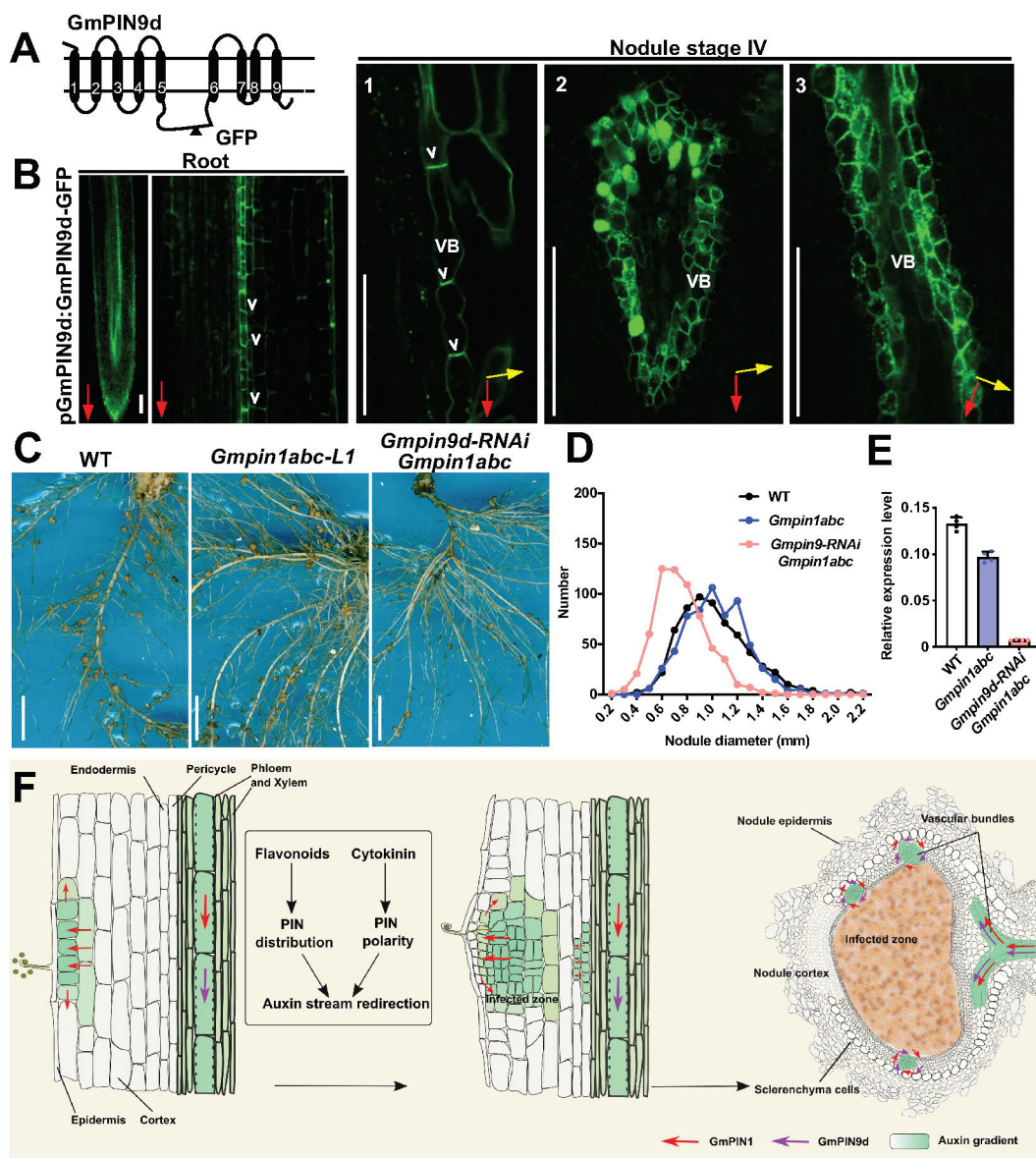
Figure 9. GmPIN9d works synergistically with GmPIN1 to coordinate nodule enlargement.

(A) GmPIN9d-GFP construct was established based on the insertion position of GFP in the hydrophilic loop.

(B) *pGmPIN9d:GmPIN9d-GFP* construct was introduced in WT soybean plant by hairy root transformation, and the positive roots were infected by *BXYD3*. Roots and nodules were collected at 14dpi for sectioning, and GmPIN9d-GFP signal was visualized. Cells with polar GmPIN9d was highlighted by white arrows. The numbered images showed 5×enlarged views (red boxes framed) in Supp.Fig. 11G images. Root tip growth directions were labelled by red arrows, and directions of nodule apex were indicated by yellow arrows. VB, vascular bundle.

(C-E) WT and *Gmpin1abc* mutants were transformed by *Gmpin9d-RNAi* construct (with dTomato-tag). The positive hairy roots were selected based on fluorescence and further infected by *BXYD3*, and the profile of nodule diameter was tracked in D (n=613, 618, 624) at 14dpi. Expression level of *GmPIN9d* was detected by RT-qPCR (E, n=4), *Gmactin11* was used as the normalization gene (E).

(F) Model for polar auxin transport in nodule primordium and developing nodule. Stage I, GmPIN1 mainly directs auxin flow to the outer cortical cells, and partial auxin flows away from outer cortical cells. Stage II and III: GmPIN1-dependent auxin stream flows towards the nodule primordium, and partial auxin is retrieved to the neighboring cells. Stage IV: auxin stream is transported within vascular bundles towards nodule via GmPIN1 and GmPIN9d-dependent polar auxin transport. Auxin accumulations are depicted in green, auxin gradient is indicated by light green, and the presumptive routes of auxin flow are depicted by arrows (red arrows: GmPIN1-mediation; purple arrows: GmPIN9d-mediation). Flavonoid and cytokinin act as the elicitors to mediate rapid auxin stream redirection.

Scale bars, 100μm (B), 1cm (C). Error bar=S.D.

Parsed Citations

Abas, L., Kolb, M., Stadlmann, J., Janacek, D.P., Lukic, K., Schwechheimer, C., Sazanov, L.A., Mach, L., Friml, J., and Hammes, U.Z. (2021). Naphthylphthalamic acid associates with and inhibits PIN auxin transporters. Proc Natl Acad Sci U S A 118.
Google Scholar: Author Only Title Only Author and Title

Adamowski, M., and Friml, J. (2015). PIN-dependent auxin transport: action, regulation, and evolution. Plant Cell 27, 20-32.
Google Scholar: Author Only Title Only Author and Title

Bai, M., Yuan, J., Kuang, H., Gong, P., Li, S., Zhang, Z., Liu, B., Sun, J., Yang, M., Yang, L., Wang, D., Song, S., and Guan, Y. (2020). Generation of a multiplex mutagenesis population via pooled CRISPR-Cas9 in soya bean. Plant Biotechnol J 18, 721-731.
Google Scholar: Author Only Title Only Author and Title

Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., and Noble, W.S. (2009). MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37, W202-208.
Google Scholar: Author Only Title Only Author and Title

Benkova, E., and Bielach, A. (2010). Lateral root organogenesis - from cell to organ. Curr Opin Plant Biol 13, 677-683.
Google Scholar: Author Only Title Only Author and Title

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591-602.
Google Scholar: Author Only Title Only Author and Title

Bozsoki, Z., Gysel, K., Hansen, S.B., Lironi, D., Kronauer, C., Feng, F., de Jong, N., Vinther, M., Kamble, M., Thygesen, M.B., Engholm, E., Kofoed, C., Fort, S., Sullivan, J.T., Ronson, C.W., Jensen, K.J., Blaise, M., Oldroyd, G., Stougaard, J., Andersen, K.R., and Radutoiu, S. (2020). Ligand-recognizing motifs in plant LysM receptors are major determinants of specificity. Science 369, 663-670.
Google Scholar: Author Only Title Only Author and Title

Brown, D.E., Rashotte, A.M., Murphy, A.S., Normanly, J., Tague, B.W., Peer, W.A., Taiz, L., and Muday, G.K. (2001). Flavonoids act as negative regulators of auxin transport in vivo in arabidopsis. Plant Physiol 126, 524-535.
Google Scholar: Author Only Title Only Author and Title

Buer, C.S., Kordbacheh, F., Truong, T.T., Hocart, C.H., and Djordjevic, M.A. (2013). Alteration of flavonoid accumulation patterns in transparent testa mutants disturbs auxin transport, gravity responses, and imparts long-term effects on root and shoot architecture. Planta 238, 171-189.
Google Scholar: Author Only Title Only Author and Title

Cai, Z., Wang, Y., Zhu, L., Tian, Y., Chen, L., Sun, Z., Ullah, I., and Li, X. (2017). GmTIR1/GmAFB3-based auxin perception regulated by miR393 modulates soybean nodulation. New Phytol 215, 672-686.
Google Scholar: Author Only Title Only Author and Title

Calvert, H.E., Pence, M.K., Pierce, M., Malik, N.S.A., and Bauer, W.D. (1984). Anatomical analysis of the development and distribution of Rhizobium infections in soybean roots. Canadian Journal of Botany 62, 2375-2384.
Google Scholar: Author Only Title Only Author and Title

Chen, X., Irani, N.G., and Friml, J. (2011). Clathrin-mediated endocytosis: the gateway into plant cells. Curr Opin Plant Biol 14, 674-682.
Google Scholar: Author Only Title Only Author and Title

Du, M., Spalding, E.P., and Gray, W.M. (2020). Rapid Auxin-Mediated Cell Expansion. Annu Rev Plant Biol 71, 379-402.
Google Scholar: Author Only Title Only Author and Title

Feraru, E., and Friml, J. (2008). PIN polar targeting. Plant Physiol 147, 1553-1559.
Google Scholar: Author Only Title Only Author and Title

Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. Nature 415, 806-809.
Google Scholar: Author Only Title Only Author and Title

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 282, 2226-2230.
Google Scholar: Author Only Title Only Author and Title

Gauthier-Coles, C., White, R.G., and Mathesius, U. (2018). Nodulating Legumes Are Distinguished by a Sensitivity to Cytokinin in the Root Cortex Leading to Pseudonodule Development. Front Plant Sci 9, 1901.
Google Scholar: Author Only Title Only Author and Title

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112, 219-230.
Google Scholar: Author Only Title Only Author and Title

Hirsch, A.M., Bhuvaneswari, T.V., Torrey, J.G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc Natl Acad Sci U S A 86, 1244-1248.

Ip, H., D'Aoust, F., Begum, A.A., Zhang, H., Smith, D.L., Driscoll, B.T., and Charles, T.C. (2001). Bradyrhizobium japonicum mutants with enhanced sensitivity to genistein resulting in altered nod gene regulation. Mol Plant Microbe Interact 14, 1404-1410.
Google Scholar: Author Only Title Only Author and Title

Kereszt, A., Li, D., Indrasumunar, A., Nguyen, C.D., Nontachaiyapoom, S., Kinkema, M., and Gresshoff, P.M. (2007). Agrobacterium rhizogenes-mediated transformation of soybean to study root biology. Nat Protoc 2, 948-952.
Google Scholar: Author Only Title Only Author and Title

Kohlen, W., Ng, J.L.P., Deinum, E.E., and Mathesius, U. (2018). Auxin transport, metabolism, and signalling during nodule initiation: indeterminate and determinate nodules. J Exp Bot 69, 229-244.
Google Scholar: Author Only Title Only Author and Title

Kuhn, B.M., Nodzynski, T., Errafi, S., Bucher, R., Gupta, S., Aryal, B., Dobrev, P., Bigler, L., Geisler, M., Zazimalova, E., Friml, J., and Ringli, C. (2017). Flavonol-induced changes in PIN2 polarity and auxin transport in the Arabidopsis thaliana rol1-2 mutant require phosphatase activity. Sci Rep 7, 41906.
Google Scholar: Author Only Title Only Author and Title

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 35, 1547-1549.
Google Scholar: Author Only Title Only Author and Title

Kurepa, J., Shull, T.E., and Smalle, J.A. (2019). Antagonistic activity of auxin and cytokinin in shoot and root organs. Plant Direct 3, e00121.
Google Scholar: Author Only Title Only Author and Title

Laffont, C., Ivanovici, A., Gautrat, P., Brault, M., Djordjevic, M.A., and Frugier, F. (2020). The NIN transcription factor coordinates CEP and CLE signaling peptides that regulate nodulation antagonistically. Nat Commun 11, 3167.
Google Scholar: Author Only Title Only Author and Title

Lang, K., Lindemann, A., Hauser, F., and Gottfert, M. (2008). The genistein stimulon of Bradyrhizobium japonicum. Mol Genet Genomics 279, 203-211.
Google Scholar: Author Only Title Only Author and Title

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.
Google Scholar: Author Only Title Only Author and Title

Lewis, D.R., and Muday, G.K. (2009). Measurement of auxin transport in Arabidopsis thaliana. Nat Protoc 4, 437-451.
Google Scholar: Author Only Title Only Author and Title

Liao, C.Y., Smet, W., Brunoud, G., Yoshida, S., Vernoux, T., and Weijers, D. (2015). Reporters for sensitive and quantitative measurement of auxin response. Nat Methods 12, 207-210, 202 p following 210.
Google Scholar: Author Only Title Only Author and Title

Liu, C.W., and Murray, J.D. (2016). The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. Plants (Basel) 5, 33.
Google Scholar: Author Only Title Only Author and Title

Livingston, D., Tuong, T., Nogueira, M., and Sinclair, T. (2019). Three-dimensional reconstruction of soybean nodules provides an update on vascular structure. Am J Bot 106, 507-513.
Google Scholar: Author Only Title Only Author and Title

Marhavy, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., and Benkova, E. (2014). Cytokinin controls polarity of PIN1-dependent auxin transport during lateral root organogenesis. Curr Biol 24, 1031-1037.
Google Scholar: Author Only Title Only Author and Title

Mashiguchi, K., Tanaka, K., Sakai, T., Sugawara, S., Kawaide, H., Natsume, M., Hanada, A., Yaeno, T., Shirasu, K., Yao, H., McSteen, P., Zhao, Y., Hayashi, K., Kamiya, Y., and Kasahara, H. (2011). The main auxin biosynthesis pathway in Arabidopsis. Proc Natl Acad Sci U S A 108, 18512-18517.
Google Scholar: Author Only Title Only Author and Title

Mathesius, U., Schlaman, H.R., Spaink, H.P., Of Sautter, C., Rolfe, B.G., and Djordjevic, M.A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. The Plant journal : for cell and molecular biology 14, 23-34.
Google Scholar: Author Only Title Only Author and Title

Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P., Chand, S., Benkova, E., Zazimalova, E., and Friml, J. (2008). Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development 135, 3345-3354.
Google Scholar: Author Only Title Only Author and Title

Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. EMBO J 17, 6903-6911.
Google Scholar: Author Only Title Only Author and Title

Murray, J.D., Karas, B.J., Sato, S., Tabata, S., Amyot, L., and Szczyglowski, K. (2007). A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. Science 315, 101-104.
  Google Scholar: Author Only Title Only Author and Title

Ng, J.L., Hassan, S., Truong, T.T., Hocart, C.H., Laffont, C., Frugier, F., and Mathesius, U. (2015). Flavonoids and Auxin Transport Inhibitors Rescue Symbiotic Nodulation in the Medicago truncatula Cytokinin Perception Mutant cre1. Plant Cell 27, 2210-2226.
  Google Scholar: Author Only Title Only Author and Title

Ng, J.L.P., and Mathesius, U. (2018). Acropetal Auxin Transport Inhibition Is Involved in Indeterminate But Not Determinate Nodule Formation. Front Plant Sci 9, 169.
  Google Scholar: Author Only Title Only Author and Title

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. Plant Cell 3, 677-684.
  Google Scholar: Author Only Title Only Author and Title

Pacios-Bras, C., Schlaman, H.R., Boot, K., Admiraal, P., Langerak, J.M., Stougaard, J., and Spaink, H.P. (2003). Auxin distribution in Lotus japonicus during root nodule development. Plant Mol Biol 52, 1169-1180.
  Google Scholar: Author Only Title Only Author and Title

Peer, W.A., Bandyopadhyay, A., Blakeslee, J.J., Makam, S.N., Chen, R.J., Masson, P.H., and Murphy, A.S. (2004). Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in Arabidopsis thaliana. Plant Cell 16, 1898-1911.
  Google Scholar: Author Only Title Only Author and Title

Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J.J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O.R., Fink, G.R., Geisler, M., Murphy, A.S., Luschnig, C., Zazimalova, E., and Friml, J. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312, 914-918.
  Google Scholar: Author Only Title Only Author and Title

Pierre-Jerome, E., Drapek, C., and Benfey, P.N. (2018). Regulation of Division and Differentiation of Plant Stem Cells. Annu Rev Cell Dev Biol 34, 289-310.
  Google Scholar: Author Only Title Only Author and Title

Popp, C., and Ott, T. (2011). Regulation of signal transduction and bacterial infection during root nodule symbiosis. Curr Opin Plant Biol 14, 458-467.
  Google Scholar: Author Only Title Only Author and Title

Qi, J., Wang, Y., Yu, T., Cunha, A., Wu, B., Vernoux, T., Meyerowitz, E., and Jiao, Y. (2014). Auxin depletion from leaf primordia contributes to organ patterning. Proc Natl Acad Sci U S A 111, 18769-18774.
  Google Scholar: Author Only Title Only Author and Title

Reid, D., Nadzieja, M., Novak, O., Heckmann, A.B., Sandal, N., and Stougaard, J. (2017). Cytokinin Biosynthesis Promotes Cortical Cell Responses during Nodule Development. Plant Physiol 175, 361-375.
  Google Scholar: Author Only Title Only Author and Title

Rightmyer, A.P., and Long, S.R. (2011). Pseudonodule formation by wild-type and symbiotic mutant Medicago truncatula in response to auxin transport inhibitors. Mol Plant Microbe Interact 24, 1372-1384.
  Google Scholar: Author Only Title Only Author and Title

Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L., Klein, M., Bailly, A., Lee, Y., Friml, J., Geisler, M., and Martinoia, E. (2008). Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses. J Biol Chem 283, 31218-31226.
  Google Scholar: Author Only Title Only Author and Title

Schaller, G.E., Bishopp, A., and Kieber, J.J. (2015). The yin-yang of hormones: cytokinin and auxin interactions in plant development. Plant Cell 27, 44-63.
  Google Scholar: Author Only Title Only Author and Title

Schiessl, K., Lilley, J.L.S., Lee, T., Tamvakis, I., Kohlen, W., Bailey, P.C., Thomas, A, Luptak, J., Ramakrishnan, K., Carpenter, M.D., Mysore, K.S., Wen, J., Ahnert, S., Grieneisen, V.A., and Oldroyd, G.E.D. (2019). NODULE INCEPTION Recruits the Lateral Root Developmental Program for Symbiotic Nodule Organogenesis in Medicago truncatula. Curr Biol 29, 3657-3668 e3655.
  Google Scholar: Author Only Title Only Author and Title

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W., Guo, H., Ljung, K., and Alonso, J.M. (2011). The Arabidopsis YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. Plant Cell 23, 3961-3973.
  Google Scholar: Author Only Title Only Author and Title

Subramanian, S., Stacey, G., and Yu, O. (2006). Endogenous isoflavones are essential for the establishment of symbiosis between soybean and Bradyrhizobium japonicum. The Plant journal : for cell and molecular biology 48, 261-273.
  Google Scholar: Author Only Title Only Author and Title

Subramanian, S., Stacey, G., and Yu, O. (2007). Distinct, crucial roles of flavonoids during legume nodulation. Trends Plant Sci 12, 282-285.

Google Scholar: Author Only Title Only Author and Title

Suzaki, T., and Kawaguchi, M. (2014). Root nodulation: a developmental program involving cell fate conversion triggered by symbiotic bacterial infection. Curr Opin Plant Biol 21, 16-22.
  Google Scholar: Author Only Title Only Author and Title

Suzaki, T., Yano, K., Ito, M., Umehara, Y., Suganuma, N., and Kawaguchi, M. (2012). Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response. Development 139, 3997-4006.
  Google Scholar: Author Only Title Only Author and Title

Teale, W.D., Pasternak, T., Dal Bosco, C., Dovzhenko, A., Kratzat, K., Bildl, W., Schworer, M., Falk, T., Ruperti, B., Schaefer, J.V., Shahriari, M., Pilgermayer, L., Li, X., Lubben, F., Pluckthun, A., Schulte, U., and Palme, K. (2021). Flavonol-mediated stabilization of PIN efflux complexes regulates polar auxin transport. EMBO J 40, e104416.
  Google Scholar: Author Only Title Only Author and Title

Turner, M., Nizampatnam, N.R., Baron, M., Coppin, S., Damodaran, S., Adhikari, S., Arunachalam, S.P., Yu, O., and Subramanian, S. (2013). Ectopic expression of miR160 results in auxin hypersensitivity, cytokinin hyposensitivity, and inhibition of symbiotic nodule development in soybean. Plant Physiol 162, 2042-2055.
  Google Scholar: Author Only Title Only Author and Title

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9, 1963-1971.
  Google Scholar: Author Only Title Only Author and Title

van Noorden, G.E., Kerim, T., Goffard, N., Wiblin, R., Pellerone, F.I., Rolfe, B.G., and Mathesius, U. (2007). Overlap of proteome changes in Medicago truncatula in response to auxin and Sinorhizobium meliloti. Plant Physiol 144, 1115-1131.
  Google Scholar: Author Only Title Only Author and Title

Viaene, T., Delwiche, C.F., Rensing, S.A., and Friml, J. (2013). Origin and evolution of PIN auxin transporters in the green lineage. Trends Plant Sci 18, 5-10.
  Google Scholar: Author Only Title Only Author and Title

Wang, Y., Chai, C., Valliyodan, B., Maupin, C., Annen, B., and Nguyen, H.T. (2015a). Genome-wide analysis and expression profiling of the PIN auxin transporter gene family in soybean (Glycine max). BMC Genomics 16, 951.
  Google Scholar: Author Only Title Only Author and Title

Wang, Y., Yang, W., Zuo, Y., Zhu, L., Hastwell, A.H., Chen, L., Tian, Y., Su, C., Ferguson, B.J., and Li, X. (2019). GmYUC2a mediates auxin biosynthesis during root development and nodulation in soybean. J Exp Bot 70, 3165-3176.
  Google Scholar: Author Only Title Only Author and Title

Wang, Y., Li, K., Chen, L., Zou, Y., Liu, H., Tian, Y., Li, D., Wang, R., Zhao, F., Ferguson, B.J., Gresshoff, P.M., and Li, X. (2015b). MicroRNA167-Directed Regulation of the Auxin Response Factors GmARF8a and GmARF8b Is Required for Soybean Nodulation and Lateral Root Development. Plant Physiol 168, 984-999.
  Google Scholar: Author Only Title Only Author and Title

Weijers, D., Sauer, M., Meurette, O., Friml, J., Ljung, K., Sandberg, G., Hooykaas, P., and Offringa, R. (2005). Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in Arabidopsis. Plant Cell 17, 2517-2526.
  Google Scholar: Author Only Title Only Author and Title

Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. Science 312, 883.
  Google Scholar: Author Only Title Only Author and Title

Wu, C., Dickstein, R., Cary, A.J., and Norris, J.H. (1996). The Auxin Transport Inhibitor N-(1-Naphthyl)phthalamic Acid Elicits Pseudonodules on Nonnodulating Mutants of White Sweetclover. Plant Physiol 110, 501-510.
  Google Scholar: Author Only Title Only Author and Title

Wu, M.F., Yamaguchi, N., Xiao, J., Bargmann, B., Estelle, M., Sang, Y., and Wagner, D. (2015). Auxin-regulated chromatin switch directs acquisition of flower primordium founder fate. Elife 4, e09269.
  Google Scholar: Author Only Title Only Author and Title

Xiao, T.T., Schilderink, S., Moling, S., Deinum, E.E., Kondorosi, E., Franssen, H., Kulikova, O., Niebel, A., and Bisseling, T. (2014). Fate map of Medicago truncatula root nodules. Development 141, 3517-3528.
  Google Scholar: Author Only Title Only Author and Title

Xiong, Y., and Jiao, Y. (2019). The Diverse Roles of Auxin in Regulating Leaf Development. Plants (Basel) 8, 243.
  Google Scholar: Author Only Title Only Author and Title

Xu, J., and Scheres, B. (2005). Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. Plant Cell 17, 525-536.
  Google Scholar: Author Only Title Only Author and Title

Yamaguchi, N., Wu, M.F., Winter, C.M., Berns, M.C., Nole-Wilson, S., Yamaguchi, A., Coupland, G., Krizek, B.A., and Wagner, D. (2013). A

molecular framework for auxin-mediated initiation of flower primordia. Dev Cell 24, 271-282.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>

Yang, H., Wang, Y., Li, L., Li, F., He, Y., Wu, J., and Wei, C. (2019). Transcriptomic and Phytochemical Analyses Reveal Root-Mediated Resource-Based Defense Response to Leaf Herbivory by Ectropis oblique in Tea Plant (Camellia sinensis). J Agric Food Chem 67, 5465-5476.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>

Zhang, J., Subramanian, S., Stacey, G., and Yu, O. (2009). Flavones and flavonols play distinct critical roles during nodulation of Medicago truncatula by Sinorhizobium meliloti. The Plant journal : for cell and molecular biology 57, 171-183.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>

Zhang, J., Nodzynski, T., Pencik, A, Rolcik, J., and Friml, J. (2010). PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. Proc Natl Acad Sci U S A 107, 918-922.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>

Zhang, X., Huang, X., Li, Y., Tao, F., Zhao, Q., and Li, W. (2021). Polar auxin transport May Be responsive to specific features of flavonoid structure. Phytochemistry 185, 112702.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>

Zhao, Y. (2018). Essential Roles of Local Auxin Biosynthesis in Plant Development and in Adaptation to Environmental Changes. Annu Rev Plant Biol 69, 417-435.

Google Scholar: <u>Author Only</u> <u>Title Only</u> <u>Author and Title</u>